University of Alberta

Identification of a Determinant of High Affinity Agonist Binding of γ-Aminobutyric Acid Type A Receptors and the Role of High Affinity Agonist Binding in Desensitization

by

John Glen Newell ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Department of Pharmacology

Edmonton, Alberta

Fall 2000

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-59643-5

Canadä

UNIVERSITY OF ALBERTA

September 7, 2000

Faculty of Graduate Studies and Research
University of Alberta
Edmonton, Alberta

To whom it may concern:

We, the undersigned, do hereby grant permission to J. Glen Newell to use the material entitled "Tyrosine 62 of the γ-aminobutyric acid type A receptor β2 subunit is an important determinant of high affinity agonist binding" in modified form as Chapter 2 of his doctoral thesis. A version of this chapter has been published in *The Journal of Biological* Chemistry (275: 14198-14204).

Sincerely,

Dr. Martin Davies
Assistant Professor
Department of Pharmacology
University of Alberta

Dr. Alan N. Bateson
Associate Professor
Department of Pharmacology
University of Alberta

Susan MJ Dunn

Dr. Susan M.J. Dunn
Professor
Department of Pharmacology
University of Alberta

Department of Pharmacology
Faculty of Medicine and Dentistry

9-70 Medical Sciences Building • University of Alberta • Edmonton • Canada • T6G 2H7
Telephone: (780) 492-3575 • Fax: (780) 492-4325
www.ualberta.ca

UNIVERSITY OF ALBERTA

September 7, 2000

Faculty of Graduate Studies and Research
University of Alberta
Edmonton, Alberta

To whom it may concern:

We, the undersigned, do hereby grant permission to J. Glen Newell to use the material entitled "Identification of a domain of the γ-aminobutyric acid type A receptor that alters the sensitivity to GABA and benzodiazepine site ligands" in modified form as Chapter 4 of his doctoral thesis. A version of this chapter has been submitted to the *Journal of Neuroschemistry*.

Sincerely,

Dr. Martin Davies
Assistant Professor
Department of Pharmacology
University of Alberta

Susan MJ Dunn

Dr. Susan M.J. Dunn
Professor
Department of Pharmacology
University of Alberta

Department of Pharmacology
Faculty of Medicine and Dentistry

9-70 Medical Sciences Building • University of Alberta • Edmonton • Canada • T6G 2H7
Telephone: (780) 492-3575 • Fax: (780) 492-4325
www.ualberta.ca

UNIVERSITY OF ALBERTA

September 7, 2000

Faculty of Graduate Studies and Research
University of Alberta
Edmonton, Alberta

To whom it may concern:

We, the undersigned, do hereby grant permission to J. Glen Newell to use the material entitled "Characterization of the interaction of zopiclone with γ-aminobutyric acid type A receptors" in modified form as Chapter 5 of his doctoral thesis. A version of this chapter has been accepted for publication and will appear in *Molecular Pharmacology* (volume 54) in October 2000.

Sincerely,

Dr. Martin Davies
Assistant Professor
Department of Pharmacology
University of Alberta

Dr. Ian L. Martin
Professor
Pharmacology
Aston University, United Kingdom

Jason Derry

Mr. Jason Derry
Provisional Ph.D. Candidate
Department of Pharmacology
University of Alberta

Susan MJ Dunn

Dr. Susan M.J. Dunn
Professor
Department of Pharmacology
University of Alberta

Department of Pharmacology
Faculty of Medicine and Dentistry

9-70 Medical Sciences Building • University of Alberta • Edmonton • Canada • T6G 2H7
Telephone: (780) 492-3575 • Fax: (780) 492-4325
www.ualberta.ca

UNIVERSITY OF ALBERTA

September 7, 2000

Faculty of Graduate Studies and Research
University of Alberta
Edmonton, Alberta

To whom it may concern:

I, the undersigned, do hereby grant permission to J. Glen Newell to use the material entitled "A critical element of the high affinity agonist binding domain of γ-aminobutyric type A receptor stabilizes the desensitzed state" in modified form as Chapter 3 of his doctoral thesis. A version of this chapter has been submitted to the *Journal of Neuroschemistry*.

Sincerely,

Susan MJ Dunn

Dr. Susan M.J. Dunn
Professor
Department of Pharmacology
University of Alberta

Department of Pharmacology
Faculty of Medicine and Dentistry

9-70 Medical Sciences Building • University of Alberta • Edmonton • Canada • T6G 2H7
Telephone: (780) 492-3575 • Fax: (780) 492-4325
www.ualberta.ca

University of Alberta

Library Release Form

Name of Author: John Glen Newell

Title of Thesis: Identification of a Determinant of High Affinity Agonist Binding of γ-Aminobutyric Acid Type A Receptors and the Role of High Affinity Agonist Binding in Desensitization

Degree: Doctor of Philosophy

Year this Degree Granted: 2000

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as herein before provided, neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without with author's prior written permission.

Glen Newell

#2510, 8210 – 111 Street
Edmonton, AB
T6G 2C7

Date: September 20, 2000.

ABSTRACT

The γ-aminobutyric acid type A receptor (GABA$_A$R) carries high (K_D = 5-30 nM) and low (K_D = 0.1–1.0 μM) affinity binding sites for agonists and high affinity binding sites for benzodiazepines. This thesis describes the interactions of γ-aminobutyrate, muscimol, Ro15-4513 and zopiclone with their respective binding sites.

A specific amino acid residue in the GABA$_A$R β2 subunit has been implicated in high affinity agonist binding. Tyrosine residues at positions 62 and 74 were mutated to phenylalanine or serine and the effects on ligand binding and channel activation were determined. None of the mutations altered benzodiazepine binding or allosteric coupling properties of the receptor. Although mutations at position 74 had little effect on [^{3}H]muscimol binding, the Y62F substitution decreased the affinity of the high affinity site ~6-fold, while the Y62S substitution led to a loss of detectable high affinity binding. The potencies for both agonists were decreased 2-fold (Y62F) and 6-fold (Y62S) in ion-channel activation studies. It is therefore concluded that Tyr-62 of the β2 subunit is an important determinant of high affinity agonist binding.

To define the functional relevance of the high affinity agonist binding site(s), desensitization phenomena were also investigated. Using the *Xenopus* expression system, the concentration-dependence of desensitization was a function of both duration of agonist-preperfusion and frequency of agonist application. Expression of Y62F and Y62S increased the IC$_{50}$ values for agonist-induced desensitization ~2-fold and >5-fold, respectively. Lack of measurable high affinity binding (Y62S) facilitated recovery from the desensitized state during continuous agonist perfusion. This suggests that the role of the high affinity-binding site is to stabilize the desensitized state once it has been formed.

The interaction of zopiclone, a sedative-hypnotic, with the $GABA_AR$ has been characterized and the localization of the labeling of the $\alpha 1$ subunit by $[^3H]$Ro15-4513 has been refined. Mutagenesis of H101 of the $\alpha 1$ subunit suggests that zopiclone interacts with $GABA_AR$ in the same fashion as diazepam and flunitrazepam. Homologue and alanine-scanning mutagenesis of the putative extracellular loop between TMII and TMIII of the $\alpha 1$ subunit suggest that the region defined by Leu-276, Pro-277 and Val-279 is important for agonist sensitivity.

Chì mu gun dàil an t-aite 's an d'rugadh mi;
Cuirear orm fàilte' 's a' chànain a thuigeas mi;
Gheibh mi an aoidh agus gràdh 'nuair ruigeam,
Nach reicinn air thunnachan òir.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Identification of a Determinant of High Affinity Agonist Binding of γ-Aminobutyric Acid Type A Receptors and the Role of High Affinity Agonist Binding in Desensitization submitted by John Glen Newell in partial fulfillment for the requirements for the degree of Doctor of Philosophy.

Susan MJ Dunn

Dr. Susan M.J. Dunn, Supervisor
Professor, Department of Pharmacology

Dr. Alan N. Bateson, Co-Supervisor
Associate Professor, Department of Pharmacology

William F. Dryden

Dr. William F. Dryden, Supervisory Committee
Professor, Department of Pharmacology

Dr. James W. Young, Supervisory Committee
Professor, Department of Physiology

AClanachan

Dr. Alexander S. Clanachan, Examiner & Chair
Professor & Chair, Department of Pharmacology

Dr. David S. Weiss, External Examiner
Associate Professor, Department Of Neurobiology,
University of Alabama, Birmingham, USA

To my parents, John and Carole.

ACKNOWLEDGEMENTS

There are many individuals to whom I am grateful for their contributions to the research described within this dissertation. I am indebted to my supervisor, Dr. Susan Dunn, for her patience and words of wisdom over the past five years. It has been a pleasure to study in her lab. I would like to thank my co-supervisor, Dr. Alan Bateson, for the training in molecular biological techniques without which I would not have been able to embark on this journey. I sincerely thank Dr. Martin Davies for his daily contributions to my graduate training, both technical and intellectual, and for teaching me to surmount the inherent challenges of research.

I thank the members of my supervisory committee, Dr. Bill Dryden and Dr. Jim Young, for their support over the course of this degree. I am also grateful to Dr. Ian Martin for his encouragement and guidance and for reminding us all that perseverance and determination are the keys to success.

To all of my fellow students, both past and present, thanks for making my time at the University of Alberta memorable. I thank Mark Hildebrand for years of friendship in the curling rinks of Alberta and beyond and for helping me to achieve my goals, both personal and professional. I also thank Angie McKenna for reminding what it means to be a Maritimer.

I am grateful to Rick Thuynsma, Eugene Chomey, Brian Tancowny, and Christine Selzler for their expert technical assistance and to all of the faculty and staff of the Department of Pharmacology for their contributions to my graduate programme over the years. I thank Judy Deuel for all that she has done for me over the years. I especially thank Joy Pedersen for her assistance with the preparation of this manuscript.

The GABA$_A$R receptor subunit cDNA clones used in the mutagenesis studies were kindly provided by Dr. David S. Weiss.

Scholarship support was provided by the Natural Sciences and Engineering Research Council (NSERC) of Canada, the Alberta Heritage Foundation for Medical Research (AHFMR), the NeuroScience Canada Foundation and the University of Alberta.

TABLE OF CONTENTS

Page

CHAPTER 1 – Introduction 1

General Overview........ 2

GABA$_A$ Receptors (GABA$_A$R): Overview........ 2

GABA$_A$ Receptor Composition, Stoichiometry and Subunit Arrangement........ 4

GABA$_A$R Subunit Assembly........ 5

Heterologous Expression........ 9

Molecular Architecture of the Nicotinic Acetylcholine Receptor Binding Sites: Evolution of the Recognition Model........ 14

Glycine and 5HT$_3$ Receptors........ 18

Subunit Interfaces form Binding Sites for GABA$_A$R Agonists and Benzodiazepine Ligands........ 20

Benzodiazepine Site: The α-γ Interface........ 21

Low Affinity GABA Recognition Site: β-α Interfaces........ 23

Other Binding Sites of GABA$_A$R........ 24

Heterogeneity of Equilibrium Binding: Agonists/Antagonists........ 27

Functional Roles of Low and High Affinity Agonist Binding Sites of GABA$_A$R........ 31

Models of Receptor Activation and Desensitization........ 33

Aims of the Present Studies........ 36

Bibliography........ 56

CHAPTER 2 - Tyrosine 62 Of The γ-Aminobutyric Acid Type A Receptor β2 Subunit Is An Important Determinant Of High Affinity Agonist Binding 94

Introduction........ 95

Materials and Methods........ 98

- Site-Directed Mutagenesis 98
- Transient Transfection and Cell Membrane Preparation 98
- Equilibrium Binding Assays 99
- Expression in Oocytes and Two-Electrode Voltage Clamp Analysis 100
- Data Analysis 100
- Statistical Analysis 101

Results........ 103
    Equilibrium Binding Assays 103
    Two-Electrode Voltage Clamp Analysis 105
Discussion........ 107
Bibliography........ 144
CHAPTER 3 – A Critical Element Of The High Affinity Agonist-Binding Domain Stabilizes the Desensitized State of GABA_A Receptors 153
Introduction........ 154
Materials and Methods........ 157
    Site-Directed Mutagenesis 157
    cRNA Transcript and Oocyte Preparation 157
    Two-Electrode Voltage Clamp 157
    Pre-perfusion Protocols 157
    Statistical Analyses 159
Results........ 160
    Agonist-Induced Desensitization 160
    Optimization of Experimental Conditions to Investigate Desensitization 160
    Desensitization of GABA_AR Carrying Mutations of Y62 of the β2 Subunit 162
    Effect of Agonist Concentration of Receptor Desensitization 162
    Receptors Carrying the β2 Subunit Y62S Mutation do not Maintain the Desensitized State 162
Discussion........ 164
Bibliography........ 181
CHAPTER 4 - Mutagenesis Of The GABA_A Receptor α1 Subunit Reveals A Domain That Affects Sensitivity To GABA And Benzodiazepine Site Ligands 185
Introduction........ 186
Materials and Methods........ 188
    Site-Directed Mutagenesis 188
    Cell Culture and Transient Transfection 188
    Equilibrium Binding Assays 188
    Expression in Oocytes and Two-Electrode Voltage Clamp Analysis 189

Data and Statistical Analysis 191
Results.. 191
Equilibrium Binding Assays 191
Two-Electrode Voltage Clamp Analysis 191
Discussion.. 193
Bibliography.. 213
CHAPTER 5 - Characterization Of The Interaction Of Zopiclone With γ-Aminobutyric Acid Type$_A$ Receptors 217
Introduction.. 218
Materials and Methods... 221
Site-Directed Mutagenesis 221
Cell Culture and Transient Transfection 221
Radioligand Binding Assays 221
Photoshift Experiments 222
Electrophysiology 222
Data and Statistical Analyses 223
Results.. 224
The Effects of Photolabeling on the Recognition of Flunitrazepam and Zopiclone 224
The Effects of Zopiclone and Diazepam Binding of Substitutions at Histidine 101 of the α1 Subunit 224
The Influence of γ Subunits on Zopiclone and Flunitrazepam Recognition 225
Discussion.. 226
Bibliography.. 248
CHAPTER 6 – General Discussion 254
General Discussion... 255
Conclusions... 260
Future Directions.. 263
Bibliography.. 267

LIST OF TABLES

		Page
Table 1-1	Relative equilibrium dissociation constants (K_D) and binding densities (B_{max}) for [^{3}H]GABA or [^{3}H]muscimol binding at high (H) and low (L) affinity sites.	55
Table 2-1	The effects of amino acid substitutions in the β2 subunit of $\alpha1\beta2\gamma2_L$ recombinant $GABA_A$ receptors on high (H) and low (L) affinity [^{3}H]muscimol binding.	140
Table 2-2	The effects of amino acid substitutions in the β2 subunit of $\alpha1\beta2\gamma2_L$ recombinant $GABA_A$ receptors on the displacement of [^{3}H]muscimol by a number of $GABA_A$R ligands.	141
Table 2-3	Potentiation of [^{3}H]flunitrazepam (2 nM) binding by GABA.	142
Table 2-4	Concentration-response data for (A) GABA and (B) muscimol activation of wild type and mutant receptors expressed in *Xenopus* oocytes.	143
Table 3-1	The effects of β2 subunit mutations on the concentration-dependence of agonist-induced desensitization.	180
Table 4-1	The affinity of [^{3}H]Ro15-4513 for wild-type receptors compared with those containing mutated α1 subunits.	210
Table 4-2	Concentration-response data for GABA mediated chloride flux from recombinant $GABA_A$ receptors expressed in *Xenopus* oocytes.	211
Table 4-3	A comparison of the potency and efficacy of benzodiazepine site ligands at wild-type and V279A receptors.	212
Table 5-1	Photolabeling-induced shifts in affinity for Ro15-1788, zopiclone and flunitrazepam.	245
Table 5-2	K_I values for zopiclone and diazepam binding at $GABA_A$ receptors containing wild-type and mutant α subunits.	246
Table 5-3	Concentration-response data for flunitrazepam and zopiclone on GABA mediated current in recombinant α1β2γ2 or α1β2γ3 $GABA_A$R expressed in *Xenopus* oocytes.	247

LIST OF FIGURES

Page

Figure 1-1 General schematic of (A) the structure of a GABA$_A$R subunit and (B) linearized model of a generic subunit. 40

Figure 1-2 Model of the binding site of the nAChR. 42

Figure 1-3 Partial amino acid sequence alignment for the α1, β2, and γ2 subunits of the rat GABA$_A$R, α, γ and δ subunits of the *Torpedo* nAChR subunits, α and β subunits of the rat GlyR, and the A subunit of the rat 5HT$_3$R from (A) "loop" A, (B) "loop" B, and (C) "loop" C. 44

Figure 1-4 Partial amino acid sequence alignment for the α1, β2, and γ2subunits of the rat GABA$_A$R, the α, γ and δ subunits of the *Torpedo* nAChR subunits, α and β subunits of the rat GlyR, and the A subunit of the rat 5HT$_3$R from (A) "loop" D, (B) "loop" E, and (C) "loop" F. 46

Figure 1-5 Model of the benzodiazepine-binding site at the interface of adjacent α–γ subunits of the GABA$_A$R. 48

Figure 1-6 Model of the low affinity-binding site at the interface of adjacent β–α subunits of the GABA$_A$R. 50

Figure 1-7 Models of GABA$_A$R activation and desensitization as proposed by (A) del Castillo and Katz (1957) and (B) Jones *et al.* (1998). 52

Figure 1-8 Models of GABA$_A$R activation and desensitization as proposed by (A) Heidmann and Changeux (1980) and (B) Raftery *et al.* (1983). 54

Figure 2-1 Model of a recombinant GABA$_A$R showing putative stoichiometry and subunit arrangement. 113

Figure 2-2 Partial amino acid sequence alignment for the α1-, β2-, and γ2-subunits of the rat GABA$_A$R, γ and δ subunits of the *Torpedo* nAChR subunits, α subunit of the rat GlyR, and the A subunit of the rat 5HT$_3$R from the amino acid sequence of "loop" D. 115

Figure 2-3 Chemical structures of the agonists, GABA and muscimol, and the competitive antagonist, bicuculline methochloride. 117
Figure 2-4 Saturation curves for [^{3}H]Ro15-4513. 119
Figure 2-5 Equilibrium binding data for [^{3}H]muscimol in WT receptors. 121
Figure 2-6 Equilibrium binding data for [^{3}H]muscimol in $\alpha1\beta2(Y74F)\gamma2_L$ receptors. 123
Figure 2-7 Equilibrium binding data for [^{3}H]muscimol in $\alpha1\beta2(Y74S)\gamma2_L$ receptors. 125
Figure 2-8 Equilibrium data for [^{3}H]muscimol binding to $\alpha1\beta2(Y62F)\gamma2_L$ and $\alpha1\beta2(Y62S)\gamma2L$ mutant receptors. 127
Figure 2-9 Competition curves for displacement of [^{3}H]muscimol by bicuculline from recombinant receptors expressing WT, Y62F, Y74F, and Y74S β2 subunits. 129
Figure 2-10 Concentration-response curve for potentiation of 2 nM [^{3}H]flunitrazepam binding to $\alpha1\beta2\gamma2$ receptors. 131
Figure 2-11 Concentration-dependence of agonist activated Cl$^-$ conductances for recombinant GABA$_A$R in which WT, Y62F, and Y62S β2 subunits were expressed. 133
Figure 2-12 Representative traces for GABA activation of recombinant GABA$_A$R expressing WT (A), Y62F (B) and Y62 (C) β2 subunits. 135
Figure 2-13 Effects of bicuculline on GABA gated chloride conductance in recombinant GABA$_A$R expressing WT, Y62F, and Y62 β2 subunits. 137
Figure 2-14 Representative traces for bicuculline antagonism of GABA gated chloride conductance in recombinant GABA$_A$R expressing WT (A), Y62F (B) and Y62 (C) β2 subunits. 139
Figure 3-1 Representative recordings from application of GABA to recombinant $\alpha1\beta2\gamma2$ receptors expressed in *Xenopus* oocytes. 169
Figure 3-2 (A) Inhibitory effects of GABA perfusion on GABA (1 mM)

mediated Cl⁻ conductances in recombinant GABA$_A$R containing WT β2 subunits. (B) Effect of duration of agonist perfusion on GABA gated current in α1β2γ2 receptors. 171

Figure 3-3 Inhibitory effects of GABA perfusion on GABA-mediated (1 mM) Cl⁻ conductances in recombinant GABA$_A$R containing Y62F (A) and Y62S (B) β2 subunits. 173

Figure 3-4 Concentration-dependence of agonist-induced desensitization in recombinant α1β2γ2 GABA$_A$R expressing (A) WT, (B) Y62F and (C) Y62S β2 subunits. 175

Figure 3-5 Representative traces from recovery experiments for WT (A) and Y62S (B) containing receptors that are pre-perfused with an agonist concentration of 3 μM. 177

Figure 3-6 Schematic of the allosteric transitions of a recombinant GABA$_A$R, carrying 2 high affinity binding sites and 2 low affinity binding sites. 179

Figure 4-1 Residues 273 to 283 in the putative extracellular loop between TMII and TMIII of the rat α1 subunit. 197

Figure 4-2 Concentration response relationships for GABA with wild-type and mutant receptors S275T, L276A, P277A, V279A, A280S and Y281F. 199

Figure 4-3 Representative traces showing GABA activation of recombinant GABA$_A$R expressing WT (A), S275T (B), L276A (C) and P277A (D) α1 subunits. 201

Figure 4-4 Representative traces showing GABA activation of recombinant GABA$_A$R expressing V279A (A), A280S (B) and Y281F (C) α1 subunits. 203

Figure 4-5 The V279A mutation does not change the efficacy of benzodiazepine site ligands. 205

Figure 4-6 Representative traces for the V279A mutation, illustrating the pharmacological actions of benzodiazepine ligands on GABA- 207 gated current.

Figure 4-7 The effects of flunitrazepam on GABA concentration-response curves for wild-type and V279A receptors. 209

Figure 5-1 Chemical structures of (A) diazepam, (B) flunitrazepam and (C) zopiclone 232

Figure 5-2 Competition curves showing the displacement of $[^3H]$Ro15-4513 by increasing concentrations of zopiclone from membranes prepared from cells expressing wild type, H101F, H101Q and H101Y α subunits coexpressed with β2 and γ2 subunits. 234

Figure 5-3 Competition curves showing the displacement of $[^3H]$Ro15-4513 by increasing concentrations of diazepam from membranes prepared from cells expressing wild type, H101F, H101Q and H101Y α subunits coexpressed with β2 and γ2 subunits. 236

Figure 5-4 The effect of the γ3 subunits on the affinities of benzodiazepine site ligands. 238

Figure 5-5 Concentration-dependence of agonist activated Cl$^-$ conductance for recombinant α1β2γ3 GABA$_A$R. 240

Figure 5-6 Concentration-response curves illustrating the potentiation of GABA mediated Cl$^-$ conductance by flunitrazepam and zopiclone with recombinant α1β2γ2 (A) and α1β2γ3 (B) GABA$_A$R expressed in *Xenopus* oocytes. 242

Figure 5-7 Orientation of diazepam, ZK93423 and zopiclone in the benzodiazepine pharmacophore of Zhang *et al.* (1995). 244

LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	analysis of variance
BES	N,N-bis(2-hydroxyethyl)-2-aminomethanesulfonic acid
BIP	binding protein
B_{max}	maximum binding density
BZ1	benzodiazepine type 1 receptor
BZ2	benzodiazepine type 2 receptor
BZ3	benzodiazepine type 3 receptor
BZD	benzodiazepine
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
cRNA	complementary ribonucleic acid
DDF	ρ-(N,N)-dimethyl-aminobenzenediazoniumfluoroborate
diazepam	7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one
DMSO	dimethylsulfoxide
DMT	dimethyl-*d*-tubocurarine
*d*TC	*d*-tubocurarine
EBOB	4'-ethynyl-4-n-propylbicycloorthobenzoate
EC_{50}	half-maximal concentration for channel activation
E_{max}	maximum response
ER	endoplasmic reticulum
flunitrazepam	5-(-2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one
flunitrazepam	flunitrazepam
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid type A receptor
GFP	green fluorescence protein
GlyR	glycine receptor
5-HT	5-hydroxytryptamine (serotonin)

5HT$_3$R	5-hydroxytryptamine type 3 receptor
HEK-293	human embryonic kidney 293
HEPES	4-(hydroxyethyl)-1-piperazineethanesulfonic acid
K$_D$	equilibrium ligand dissociation constant
K$_I$	equilibrium inhibitor dissociation constant
LGIC	ligand-gated ion channel
MBTA	4-(-*N*-maleimido)benzyltrimethylammonium
MSBD	*meta*-sulfonate benzene diazonium
MTSEA	*N*-biotinlyaminoethyl methanethiosulfonate
MTS	methanethiosulfonate
muscimol	3-hydroxy-5-aminomethylisoxazole
nAChR	nicotinic acetylcholine receptor
PMSF	phenylmethylsulfonylfluoride
PKA	protein kinase A
PKC	protein kinase C
Ro15-4513	ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5a][1,4]-benzodiazepine-3-carboxylate
Ro15-1788	8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5a][1,4]benzodiazepine-3-carboxylic acid
SCAM	substituted cysteine accessibility method
TBPS	*tert*-butylbicyclophosphorothionate
Tris	tris(hydroxymethyl)aminomethane
zopiclone	4-methyl-1-1piperazinecarboxylic acid-6-(-5-chloro-2-pyridinyl)-6,7-dihydro-7-oxo-5H-pyrrolo-[3m4-b]pyrazin-5-ylester

CHAPTER 1

Introduction

INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system (CNS) and it is localized to 20-50% of all synapses in the brain (*see* Sieghart, 1989; Hevers and Lüddens, 1998). The decarboxylation of glutamate by glutamic acid decarboxylase is the principal biosynthetic pathway for the production of GABA (Roberts and Sherman, 1992). The binding of GABA to its receptors usually produces a hyperpolarizing response (by virtue of an increase in chloride or potassium ion conductance), although there are some cases in specific neuronal populations of the hippocampus (Michelson and Wong, 1991) and during development (*see* Cherubini *et al.*, 1991) when this neurotransmitter acts as an excitatory substance. GABA elicits its responses by binding to two distinct classes of receptors, the type A ($GABA_A$) and type B ($GABA_B$) receptors, the former of which is a ligand-gated ion channel and the latter of which is a G-protein coupled receptor that is insensitive to bicuculline and, at which, baclofen is an agonist.

$GABA_A$ RECEPTORS: OVERVIEW

The nicotinic acetylcholine receptors (nAChRs), glycine receptors (GlyRs), 5-hydroxytryptamine (serotonin) type 3 receptor ($5HT_3Rs$) and γ-aminobutyric acid type A receptors ($GABA_ARs$) share 15-18% sequence identity (Schofield *et al.*, 1987) and are classified as a family of receptors on the basis of this structural homology. $GABA_ARs$ are heteropentameric protein complexes (Nayeem *et al.*, 1994), the subunits of which are arranged to form a central anion-selective channel. The amino terminal region (~220 amino acids) of these subunits contains a conserved cysteine-cysteine bond (between residues 139 and 153) and sites for asparagine (*N*)-linked glycosylation (Schofield *et al.*, 1987), including N10 and N110 of the bovine α subunit, N8, N80 and N149 of the bovine β subunit (Schofield *et al.*, 1987), and N13, N90 and N113 of the rat γ2 subunit (Pritchett *et al.*, 1989a). Furthermore, the amino-terminus contains domains for ligand recognition and subunit assembly (see below). Each subunit has a molecular size between 50-58 kDa, which varies slightly according to the specific subunit isoforms and their state of glycosylation (*see* Stephenson, 1995). On the basis of the well-characterized nAChR and the hydropathy profile of $GABA_AR$ subunits, it has been proposed that there are four membrane spanning domains (TMI, TMII, TMIII, and TMIV), which are comprised of 22-23 amino acids that are largely hydrophobic in nature (Schofield *et al.*, 1987). The second of these domains, TMII, is believed to form the lining of the lumen of the channel, the selectivity filter of which is a ring of positively charged lysine and arginine residues (Schofield *et al.*, 1987). Between TMIII and TMIV is a large intracellular loop that is the least conserved portion of the primary sequence of the $GABA_AR$ subunit proteins (Fig. 1-1A). This loop contains consensus sequences for phosphorylation by protein kinase A (PKA) (Schofield *et al.*, 1987), protein kinase C (PKC) (Moss *et al.*, 1992; Filipova *et al.*, 2000), and tyrosine kinases (Moss *et al.*, 1995), the functional significance of which remains ambiguous. Each subunit has short a carboxyl terminal tail (Schofield *et al.*, 1987), which has been unambiguously localized to the extracellular space (Connor *et al.*, 1998).

Molecular cloning has identified a number of subunit classes, many of which have multiple isoforms, including α1-6, β1-3, γ1-3, ρ1-3, δ, ε, π and θ (for review, *see* Barnard *et al.*, 1998). Further heterogeneity arises from the fact that the genes encoding several subunits undergo alternate splicing [e.g. γ2 (Whiting *et al.*, 1990), α6 (Korpi *et al.*, 1994), β4 (Bateson *et al.*, 1991), β2 (Harvey *et al.*, 1994), β3 (Kirkness and Fraser, 1993)], which generates long and short forms that differ by the insertion of a specific number of amino acids (for review, *see* Hevers and Lüddens, 1998). The pharmacological properties of a native or recombinant receptor depend on the specific complement of subunit isoforms present within the pentamer. However, of the receptor subunit classes, recombinant αβγ receptors display the full pharmacological profile associated with most native $GABA_A$ receptors (Pritchett *et al.*, 1989a), although other combinations likely exist *in vivo*. Despite the abundance of functional and biochemical information as to the molecular properties of each subunit, the physiological significance of all of the subunits remains unknown. Although theoretical considerations suggest that there could be more than 100,000 possible subunit combinations (*see* Stephenson, 1995; Hevers and Lüddens, 1998), it is unlikely that all exist *in vivo* (McKernan and Whiting, 1996). In fact, it is estimated that there may be fewer than 10 (McKernan and Whiting, 1996).

GABA$_A$ RECEPTOR COMPOSITION, STOICHIOMETRY AND SUBUNIT ARRANGEMENT

To date, the subunit composition, stoichiometry and subunit arrangement of any one native receptor has not been unequivocally defined, owing, in part, to the temporal, spatial, and developmental expression of GABA$_A$R genes and the limited quantities of protein that are available. Biochemical studies using antibodies to selected GABA$_A$R subunits demonstrate that native receptors can contain more than one α subunit. For example, α1α2, α1α3, and α2α3 subunits have been reported to coexist within a single receptor in cerebral cortex (Duggan *et al.*, 1991). Purification of GABA$_A$R from cerebellum reveals that the α6 subunit can exist within the same pentamer as α1 (Pollard *et al.*, 1993), which gives rise to cerebellar GABA$_A$R that have two different benzodiazepine binding profiles typically associated with receptors expressing either α1 or α6 subunits (Khan *et al.*, 1996). Multiple γ subunit isoforms can also be found in the same receptor; specifically, γ2 and γ3 can be co-precipitated by antibodies raised against both subunits, while the γ1 subunit does not associate with other γ subunits (Quirk *et al.*, 1994; Benke *et al.*, 1996). The existence of two β subunits (β2/β3) within the same receptor has been recently described (Li and DeBlas, 1997). More detailed studies have demonstrated that, in cerebellar granule cells, there is a subpopulation of receptors containing α6, $\gamma 2_L$, and $\gamma 2_S$ subunits (Khan *et al.*, 1994a). There is also evidence for a cerebellar GABA$_A$R that has the following subunit composition: $\alpha 1 \alpha 6 \beta 2/3 \gamma 2_L \gamma 2_S$ (Khan *et al.*, 1994b). While many other subtypes have now been shown to exist, studies suggest that the α1β2γ2 and α2β3γ2 combinations are the major receptor subtypes in the brain (Benke *et al.*, 1994).

Biochemical studies that examined receptor composition served partially to define putative receptor subtypes and, in turn, fuelled speculation as to the stoichiometry of native GABA$_A$Rs. However, none has adequately addressed the issue. The initial report of the stoichiometry of a recombinant GABA$_A$R was based on changes in mutation-induced rectification properties in which glutamate substitutions were introduced on the C-terminal and N-terminal sides of TMII of the α3, β2, and γ2 subunits (Backus *et al.*, 1993). These substitutions produced total charge changes that were readily quantifiable using electrophysiological technology. Candidate stoichiometries included $2\alpha:2\beta:1\gamma$, $1\alpha:2\beta:2\gamma$ and $2\alpha:1\beta:2\gamma$, the most favorable of which appeared to be $2\alpha:1\beta:2\gamma$ (Backus *et al.*, 1993). More recent investigations have now shown, on the basis of the apparent affinity for GABA (Chang *et al.*, 1996), antibody avidity (Tretter *et al.*, 1997) and fluorescence energy transfer experiments (Farrar *et al.*, 1999), that a stoichiometry of $2\alpha:2\beta:1\gamma$ defines the stoichiometry of $\alpha1\beta2/3\gamma2$ receptor subtypes, at least in recombinant systems. Although this stoichiometry is widely accepted and is in good agreement with immunoprecipitation data, it is also possible that there are native receptors that are better described by the model of Backus *et al.* (1993). Information about the stoichiometry and arrangement of recombinant receptors should be interpreted with caution because the environment of a given heterologous expression system is likely to be different from the native environment of these receptors (Green and Millar, 1995).

The arrangement of these subunits within the pentamer appears to be $\alpha-\beta-\alpha-\gamma-\beta$ (Tretter *et al.*, 1997) on the basis that $\alpha1\beta3\gamma2$ and $\alpha1\beta3$ receptor subtypes form pentamers, while $\alpha1\gamma2$ and $\beta3\gamma2$ combinations form hetero-oligomers of lower molecular mass (Tretter *et al.*, 1997). This arrangement is also partially corroborated by heterologous expression studies (see below), which suggest that the $\alpha1\gamma2$ combination forms a binding site for flunitrazepam, whereas the $\alpha1\beta3$ combination forms a high affinity muscimol binding site (Zezula *et al.*, 1996). Moreover, it has now been shown that the γ subunit is situated between an $\alpha1$ and $\beta3$ subunit since a truncated version of the $\gamma2$ subunit forms specific high affinity contact sites with both subunits (Klausberger *et al.*, 2000). To summarize the available data, it is likely that a major subtype is $\alpha1\beta2\gamma2$ in a stoichiometry of $2\alpha:2\beta:\gamma$ and that the clockwise arrangement of subunits in the pentamer is $\alpha1\text{-}\beta2\text{-}\alpha1\text{-}\gamma2\text{-}\beta2$.

GABA$_A$R SUBUNIT ASSEMBLY

Directly related to the question of subunit stoichiometry is GABA$_A$R assembly and the identification of residues which mediate heteromeric or homomeric oligomerization. The assembly of GABA$_A$R subunits is a dynamic process that involves subunit folding, oligomerization via specific subunit contacts, and post-translational modifications, which include glycosylation, acylation, and proteolytic cleavage (Hurtley and Helenius, 1989). Of these stages, glycosylation seems to affect differentially the assembly process of particular proteins. Mutagenesis of putative glycosylation sites of the $\alpha1$ subunit of the $GABA_AR$ appears not to affect assembly and cell surface expression of recombinant receptors expressed in *Xenopus* oocytes, but significantly impairs cell surface expression in HEK-293 cells (Buller *et al.*, 1994). This is in excellent agreement with the observations of Connolly *et al.* (1996) in which oligomerization of $\alpha1$, $\beta2$, and $\gamma2_L$ subunits occurred independently of *N*-linked glycosylation, although the efficiency of translocation was reduced. Although little is known about the molecular mechanisms underlying these processes, the development of heterologous expression techniques has permitted the elucidation of steps within the secretory pathway of membrane-bound proteins (for review, *see* Hurtley and Helenius, 1989). The endoplasmic reticulum (ER) provides an ideal environment for protein assembly, given its high intracellular calcium ion concentration and neutral pH (Helenius *et al.*, 1995). The accepted model of protein assembly is that newly synthesized proteins reach an intermediate state at which point much of the secondary structure has formed, although the tertiary structure remains ill defined. Proteins in this stage are flexible because they need to adopt their final conformations and make contact with the subunits next to which they are found in the mature pentamer. Oligomerization is a requisite for intracellular transport to the plasma membrane (Hurtley and Helenius, 1989). The subsequent stages of $GABA_AR$ assembly involve chaperone proteins such as calnexin and binding protein (BIP) (Connolly *et al.*, 1996), both of which facilitate proper folding by interactions with specific domains of the polypeptide chain. In doing so, these proteins also subserve other physiological functions such as the prevention of protein aggregation and misfolding and the retention of unassembled proteins (Helenius *et al.*, 1995).

While little is known about the specifics of $GABA_AR$ subunit assembly, studies of the nAChR have provided some details which are likely to represent general mechanisms by which the subunits of all ligand gated ion channels operate. The assembly process has been reported to be slow and inefficient for the nAChR (Ross *et al.*, 1991). Before complete oligomerization, it is predicted that subunits establish intersubunit contacts and their failure to do so likely leads to retention within the endoplasmic reticulum and their subsequent degradation (Pelham, 1989). The most widely accepted model for nAChR assembly is described by the maturation of the α subunits, and their assembly with γ and δ subunits, such that the α-γ and α-δ heterodimers later associate with the β subunit to form the mature protein (for review, *see* Green and Millar, 1995). This is supported by the fact that co-expression of nAChR subunits shows that $\alpha\gamma$ and $\alpha\delta$ combinations form such heterodimers, whereas no significant dimer is formed on co-expression of $\alpha\beta$ subunits (Saedi *et al.*, 1991). Clearly, this specific association requires that the α subunit be able to distinguish the γ and δ subunits from the β subunit. Alternatively, it has been proposed that $\alpha\beta\gamma$ trimers form, followed by the addition of the δ subunit and the association of the remaining α subunit (Green and Claudio, 1993).

To elucidate potential mechanisms of $GABA_AR$ assembly, investigators have relied heavily on heterologous expression systems wherein single subunits, combinations of two subunits, and combinations of three subunits are studied for their ability to reach the plasma membrane (by cell surface fluorescence) and to form oligomers (on the basis of their sedimentation coefficients). In particular, the ability to add a reporter molecule (e.g. green fluorescence protein, GFP) to a $GABA_AR$ subunit without altering the functional profile of the receptors has facilitated the study of receptor trafficking and cell surface expression. The $\alpha1\beta2$ and $\alpha1\beta2\gamma2_L$ combinations produce functional cell surface receptors, while $\alpha1\gamma2_L$ and $\beta2\gamma2_L$ are retained within the endoplasmic reticulum (Connolly *et al.*, 1996). Additionally, $\alpha1$, $\beta2$, or $\gamma2_L$ subunits, when expressed alone, fail to form cell surface receptors (Connolly *et al.*, 1996; Gorrie *et al.*, 1997; Connolly *et al.*, 1999), although $\gamma2_S$ is reported to form non-functional cell surface receptors (Connolly *et al.*, 1999). This is in good agreement with the observation that $\alpha1\beta3\gamma2$ and $\alpha1\beta3$ combinations form pentameric receptors, whereas while $\beta3\gamma2$ and $\alpha1\gamma2$ predominantly from heterodimers and are largely retained in the ER (Tretter *et* al., 1997). A subsequent study using GFP tagged subunits produced similar results, with cell surface expression of $\alpha1\beta2$ or $\alpha1\beta2\gamma2$ subunit combinations, but not the $\alpha1\gamma2$ combination or the $\alpha1$ subunit alone (Connor *et al.*, 1998). These results clearly demonstrated the importance of the $\beta2$ subunit in the efficient assembly of oligomers, although the $\beta2$ subunit, when expressed alone, appears not to reach the plasma membrane (Taylor *et al.*, 1999). This further underscores the complexity of the receptor assembly process and the requirement of these subunits to contact all correct neighbouring subunits for functional cell surface expression.

Specific residues that mediate homomeric and heteromeric expression have been identified by generating chimeric proteins of the β2 and β3 subunits, the latter of which are able to form functional cell surface receptors in certain expression systems (Taylor *et al.*, 1999), but not in others (Gorrie *et al.*, 1997). Functional surface receptors composed exclusively of the β3 subunit have been shown to migrate with a sedimentation coefficient of 9S, which is indicative of a pentameric protein complex. On the other hand, β2 homo-oligomers migrate with a coefficient of 5S, which reflects a reduced state of oligomerization (Tretter *et al.*, 1997). The use of silent reporter epitopes, combined with the generation of chimeric proteins and site-directed mutagenesis allowed for the identification of G171, K173, E179, and R180 of the β3 subunits as residues which form contact points with other β subunits and with the γ2 subunit, but not with the α1 subunit (Taylor *et al.*, 1999). Subsequently, the same authors demonstrated that Q67 of the α1 subunit was required for the oligomerization of α1 and β3 subunits, but did not affect the production of α1-γ2 complexes (Taylor *et al.*, 1999). Invariant tryptophans (W69 and W94), when mutated, prevent receptors from forming pentamers as determined by sedimentation coefficients (Srinivassan *et al.*, 1999). Finally, the most recent data on receptor assembly have shown that specific domains of the γ2 subunit contact the α1 and β3 subunits. Specifically, amino acid residues 91-104 and residues 83-90 of the γ2 subunit make contact with the α1 and β3 subunits, respectively (Klausberger *et al.*, 2000).

While much of the picture has yet to emerge, these data, taken together, suggest (1) that there are preferred combinations of subunits for the production of functional cell surface receptors, (2) that specific domains of each subunit mediate contact with other subunits, (3) that domains of the β subunit that contact the α subunit are different from those which contact the γ subunit and (4) that oligomerization is a prerequisite for cell surface expression. This is not surprising since the formation of the pentamer with a very defined arrangement of subunits appears to be critical for the generation of cell surface receptors possessing all of the basic pharmacological properties of native GABA$_A$Rs. These results also illustrate the contact points between adjacent subunits lie in regions that are in close physical proximity to putative ligand binding domains. This is not unexpected since ligand recognition domains are predicted to occur at the interface of adjacent subunits (see below). Assembly studies in heterologous systems should, however, be interpreted with caution since these studies are carried out in foreign environments that differ from the cells in which GABA$_A$R assembly would normally occur (Green and Millar, 1995). This is especially true in light of the fact that the data on receptor assembly do not agree perfectly with those obtained from heterologous expression (see below).

HETEROLOGOUS EXPRESSION

Heterologous expression of cloned GABA$_A$R subunit cDNAs has permitted the characterization of specific GABA$_A$R subtypes and has addressed subunit requirements for the formation of binding sites. These studies have also produced information as to the contribution of subunit variants for the potency of benzodiazepine ligand binding, muscimol binding and GABA-gated chloride conductance. These expression studies have therefore provided powerful information on the pharmacological properties of various receptor subtypes.

These techniques confirmed the long suspected heterogeneity of benzodiazepine receptors that had been presumed on the basis of the displacement of benzodiazepine ligands by non-benzodiazepine compounds in specific brain regions. This was first noted for receptors in hippocampus where compounds such as the triazolopyridazine, CL218872, and the β-carboline, β-CCE, displaced other classical benzodiazepines such as flunitrazepam with lower affinity than receptors in cerebellum and with apparent multiple affinities as suggested by the shallow slopes of the competition curves (Nielsen and Braestrup, 1980; Squires *et al.*, 1979). This suggested that these compounds, unlike classical benzodiazepines, could discriminate among benzodiazepine receptors, which necessitated the classification of BZ1 and BZ2 receptors, having respectively high and low affinity for CL218872, 2-oxoquazepam, and certain β-carbolines (*see* Pritchett *et al.*, 1989b; Doble and Martin, 1992 and Lüddens and Wisden, 1991). The chemical synthesis of zolpidem and its activity at GABA$_A$Rs revealed a third type of benzodiazepine pharmacology, termed the BZ3 receptor. BZ3 pharmacology described a population of receptors which had a very low affinity for zolpidem (Arbilla *et al.*, 1985).

The basic subunit requirements for benzodiazepine binding and for modulation of GABA-induced current by benzodiazepines are the expression of an α and a γ subunit (Im *et al.*, 1993a; Zezula *et al.*, 1996). However, it should be noted that the β2γ2 combination is reported to carry a lower affinity benzodiazepine site that does not recognize certain benzodiazepine-site ligands such as zolpidem (Im *et al.*, 1993a). This low affinity benzodiazepine site has also been reported in α1β2γ2 receptors (Dunn *et al.*, 1999), but its functional significance is not known. Of the α subunits, α4 and α6 are insensitive to classical benzodiazepines and the only compounds that recognize receptors in which these subunits are expressed are the partial inverse agonist Ro15-4513 and other negative modulators (Kleingoor *et al.*, 1991; Wieland and Lüddens, 1994; Scholze *et al.*, 1994; Davies *et al.*, 1996). All other α subunits, when expressed with at least a γ2 subunit, recognize classical benzodiazepines with high affinity. BZ1 pharmacology is defined by expression of the α1 subunit. Heterologous expression has shown that expression of α2 and α3 subunits give rise to BZ2 pharmacology (Korpi and Seeburg, 1993; Wieland and Lüddens, 1994), whereas expression of the α5 subunit leads to BZ3 pharmacology (Korpi and Seeburg, 1993; Wieland and Lüddens, 1994; Graham *et al.*, 1996). The efficacy of any benzodiazepine ligand that is used in functional studies also depends to some extent on the expression of α subunits. Specifically, expression of α4 or α6 alters the efficacy of the antagonist, Ro15-1788, and the partial inverse agonist, Ro15-4513, such that they behave as partial agonists (Im *et al.*, 1997)

The affinity of a benzodiazepine ligand is also influenced by the γ subunit present in the pentamer. Expression of the γ3 subunit reduces the affinity for the classical benzodiazepine agonists, but does not alter the affinity of Ro15-1788, or Ro15-4513 (Herb *et al.*, 1992; Hadingham *et al.*, 1995; Benke *et al.*, 1996; Graham *et al.*, 1996; Wingrove *et al.*, 1997). Expression of the γ1 subunit, on the other hand, does not shift the affinity of classical agonists, but dramatically alters the affinity for the antagonist and the partial inverse agonist (Ymer *et al.*, 1990; Benke *et al.*, 1996). In contrast, the β subunit expressed in any particular combination does not alter the binding profiles for benzodiazepine site ligands (Hadingham *et al.*, 1993), although one report has suggested that the affinity of zolpidem was lower in receptors expressing either the β1 or β3 variant as compared to the β2 subunit (Benke *et al.*, 1994).

The minimum subunit requirements for the formation of a GABA binding site are an α and a β subunit (Pregenzer *et al.*, 1993; White *et al.*, 1995; Zezula *et al.*, 1996). In fact, α1β2 (Pregenzer *et al.*, 1993), α1β3 (Zezula *et al.*, 1996), α1β2γ2 (Pregenzer *et al.*, 1993; Ebert *et al.*, 1996; Newell *et al.*, 2000), α6β2γ2 (Korpi *et al.*, 1993; Im *et al.*, 1995; Im *et al.*, 1997), α6β2 (Im *et al.*, 1995), α1β3γ2 (Ebert *et al.*, 1996; Zezula *et al.*, 1996), α2β3γ2, α3β3γ2, α5β3γ2, and α6β3γ2 (Ebert *et al.*, 1996) all form high affinity binding sites for [^{3}H]muscimol. In contrast, no high affinity muscimol binding sites are formed on the expression of β3γ2, β3, α1, or γ2 alone (Zezula *et al.*, 1996). The affinity of muscimol generally does not differ on expression of various receptor subtypes. Lüddens and Korpi (1993) addressed this issue and found that the β variants did not influence the affinity for muscimol. However, it was also noted that certain combinations have lower affinity than α1β2γ2 for muscimol including the following: α3β1γ2, α3β1γ3, and α3β3γ3 (Lüddens and Korpi, 1993). Further, unlike the benzodiazepines, expression of either the γ2 or γ3 subunit does not affect the binding affinity of muscimol or the competitive antagonists, bicuculline and SR95531 (Lüddens *et al.*, 1994), the former of which has a higher affinity for most GABA$_A$R combinations than does bicuculline (Lüddens and Korpi, 1995).

Functional assays in which GABA-mediated currents have been studied have revealed some important information as to the influence of a variety of classes of subunits on the apparent affinity for GABA. Subunit combinations of α1β2γ2 (Verdoorn *et al.*, 1990; Sigel *et al.*, 1992; Amin and Weiss, 1993; Im *et al.*, 1993a; Mihic *et al.*, 1994; Gurley *et al.*, 1995; Chang *et al.*, 1996; Amin *et al.*, 1997; Buhr *et al.*, 1997a; Buhr *et al.*, 1997b; Westh-Hansen *et al.*, 1997; Boileau and Czajkowski, 1998; Sigel *et al.*, 1999; Dunn *et al.*, 1999; Newell *et al.*, 2000), α1β2 (Schofield *et al.*, 1987; Levitan *et al.*, 1988; Pritchett *et al.*, 1988; Sigel *et al.*, 1990; Verdoorn *et al.*, 1990; Amin *et al.*, 1994; Boileau and Czajkowski, 1998), β2γ2 (Verdoorn *et al.*, 1990; Sigel *et al.*, 1990; Im *et al.*, 1993a;

Amin *et al.*, 1994; Boileau and Czajkowski, 1998), α1β1 (Levitan *et al.*, 1988; Pritchett *et al.*, 1988; Birnir *et al.*, 1995; Tierney *et al.*, 1996), α6β2γ2 (Im *et al.*, 1995; Im *et al.*, 1997), α1β1γ2 (Wafford *et al.*, 1996; Wingrove *et al.*, 1997), α2β1 (Levitan *et al.*, 1988), α3β1 (Levitan *et al.*, 1988), α4β1γ2 (Wafford *et al.*, 1996), α1β3γ2 (Fisher *et al.*, 1997), α6β3γ2 (Fisher *et al.*, 1997), α6β2γ2 (Sigel *et al.*, 2000) and α6β1γ2 (Fisher *et al.*, 1997) all produce GABA-gated current in electrophysiological studies. In contrast, no current or only minor current is elicited when the α1, α5, β1, γ2, α3, and β2 subunits are expressed alone (Pritchett *et al.*, 1988; Sigel *et al.*, 1988; Sigel *et al.*, 1990; Joyce *et al.*, 1993). Furthermore, no current is detectable from cells in which the β1γ2 or α1γ2 subunit combinations have been expressed (Sigel *et al.*, 1990; Angelotti *et al.*, 1993; Amin *et al.*, 1994; Connor *et al.*, 1998), although current has been reported from the α1γ2 combination by some investigators (Verdoorn *et al.*, 1990; Seeburg *et al.*, 1990; Draughn *et al.*, 1991; Im *et al.*, 1993a; Im *et al.*, 1993b). These data are in reasonable agreement with assembly studies that suggest that the α1γ2 combination and certain subunits, when expressed alone, do not efficiently form functional cell surface receptors (*see* Sieghart, 1995). The implication of these data is that the existence of these receptor subtypes *in vivo* is unlikely. Therefore, small detectable currents may reflect translocation of excess protein to the plasma membrane from an oversaturated ER (Connolly *et al.*, 1996).

Although it is difficult to determine the subunits present within a recombinant receptor, there are certain characteristics associated with specfic subunits. Receptors in which α1β2 subunits are expressed have a higher affinity for GABA than α1β2γ2 (Amin *et al.*, 1994; Wingrove *et al.*, 1997; Boileau and Czajkowski, 1998); both combinations have higher affinity than β2γ2 combinations (Sigel *et al.*, 1990; Amin *et al.*, 1994; Boileau and Czajkowski, 1998). Muscimol, in similar assays, is 3-4 fold more potent than GABA in mediating chloride conductance (Amin and Weiss, 1993; Newell *et al.*, 2000). However, there is no clear consensus as to the apparent affinity for GABA at these receptor combinations (*see* Hevers and Lüddens, 1998) due to differences in the choice of expression system (HEK-293 cells or oocytes), the electrophysiological method employed (patch-clamp or two-electrode voltage clamp) and the system used for the application of agonist (rapid application or gravity perfusion).

In the absence of epitope-tagged subunits, the expression of subunit combinations can only be evaluated crudely by the macroscopic constants that are derived from any given experiment. While expression of an α and a β subunit is sufficient to produce functional $GABA_A$ receptors, it is generally possible to distinguish an αβ combination from an αβγ combination on the basis of the EC_{50} values. Expression of a γ subunit can be independently verified by measuring potentiation of GABA-gated currents using a classical positive allosteric modulator. Further, the recombinant αβ $GABA_A$Rs are more sensitive to inhibition by zinc ions than are αβγ receptors (Hornstein and Akabas, 1998). As with all heterologous expression systems, it is not possible to state with certainty that all subunits have been incorporated into a single pentamer or that there are not multiple receptor populations expressed within the same cell (*see* Sieghart, 1995), although there appear to be preferred receptor subtypes such that transfection of HEK-293 cells with α1, β1, and γ2 subunit cDNA construct most often produces α1β1γ2 receptors and rarely, if ever, α1β1 receptors in the same cell (Angelotti and MacDonald, 1993) unless transfection conditions are altered such that the β1 subunit is present 4-fold in excess (Angelotti *et al.*, 1993).

On the basis of these data, some laboratories have investigated the relative potencies of GABA agonists in recombinant receptors composed of different α, β, and γ variants. To that end, a systematic evaluation of subunit variants was examined in a single system and it was discovered that all three subunit classes could alter the apparent affinity for GABA. Specifically, it was shown that α5 has a higher affinity than either α1 or α3 (Ebert *et al.*, 1994), which is in good agreement with another study in which the rank order of potency for GABA was established as α6>α5=α4>α1>α3>α2 (Ebert *et al.*, 1996; *see* also Hevers and Lüddens, 1998). The potency of GABA is also affected by differential expression of β subunits. In all cases, expression of the β3 subunit produces receptors at which GABA is more sensitive than receptors in which the β1 or β2 subunit is found (Ebert *et al.*, 1994). Further, expression of the γ3 subunit gives rise to receptors that are reproducibly more sensitive to GABA than its γ2 and γ1 containing counterparts (Ebert *et al.*, 1994) and this increase in affinity is on the order of 3-4 fold (Knoflach *et al.*, 1991). Thus, while the basic requirement for agonist-gated current is expression of αβ subunits, the apparent affinity of the receptor (at least in the active state) appears to be influenced by the quaternary structure of the receptor.

MOLECULAR ARCHITECTURE OF THE NICOTINIC ACETYLCHOLINE RECEPTOR BINDING SITES: EVOLUTION OF THE RECOGNITION MODEL

The nAChR is the prototypical LGIC, with the defined stoichiometry of $\alpha_2\beta\gamma\delta$ (Raftery *et al.*, 1983), the subunit arrangement of which is likely α–γ–α–δ–β (Czajkowski and Karlin, 1993). This widely accepted model accounts for two non-equivalent binding sites for acetylcholine and competitive antagonists at the α–γ and α–δ interfaces (Pederson and Cohen, 1990; Blount and Merlie, 1990). While the affinity for agonists is comparable at both interfaces, *d*-tubocurarine (dTC), a competitive antagonist recognizes the binding site at the α–γ interface with high affinity, whereas the α–δ interface confers low affinity for the same compound (Blount and Merlie, 1989). In contrast, the competitive antagonist, α-conotoxin M-1, recognizes the α-δ subunit interface with high affinity, while it binds to the α-γ interface with lower affinity (Sine *et al.*, 1995)

The cysteine residues αC192 and αC193 had been the first implicated in agonist binding by affinity labeling using 4-(-N-malmeido)benzyltri[^{3}H]methylammonium iodide (MBTA) (Kao *et al.*, 1984; Kao and Karlin, 1986). Further investigations of these sites employed photolabile compounds and their derivatives, which have labeled amino acid residues of nAChR subunits that contribute to the formation of recognition sites for both agonists and antagonists, at least in the desensitized state (Colquhoun, 1998). To that end, the agonist, acetylcholine mustard, labels Y93 (Cohen *et al.*, 1991), while nicotine is primarily incorporated into Y198 and, to some extent, C192 and Y190 (Middleton and Cohen, 1991) of the α subunits in addition to a tryptophan residue (W55) of the adjacent γ subunit (Chiara *et al.*, 1998). Competitive antagonists have been used to identify many other amino acids residues that may play a role in the same recognition domains. To that end, ρ–(N,N)-dimethyl-aminobenzenediazonium fluoroborate (DDF), labels Y93, W149, Y190, C192, C193 and, to a lesser extent, W86, Y151, and Y198 of the α subunit (Dennis *et al.*, 1988; Galzi *et al.*, 1990). Further, lophotoxin analogue-1, a derivative of the snake toxin, lophotoxin, labels αY190 (Abramson *et al.*, 1989), while dTC labels αY190, αC192, αY198 (Chiara and Cohen, 1997), γY111, and γY117 (Chiara and Cohen, 1999) albeit with varying degrees of efficiency. Agonists and antagonists presumably interact with a common binding site, which suggests that these classes of compounds could potentially interact with functional groups of the same amino acid residues. However, compounds of distinct chemical structure have multiple contact points, the implication of which is that subsets of amino acids within a single binding pocket may form micro-domains which discriminate among ligands. Therefore, all of the amino acid residues that appear to form a single recognition site are not structural requirements for the binding of agonists and antagonists alike. Moreover, the relative importance of each amino acid residue within a given pocket is dependent on the chemical structure of the ligand with which it interacts.

Although photolabeling provides no insight as to the functional roles of these candidate amino acid residues, mutational analyses of nAChR in mouse and *Torpedo* have provided data on the importance of the relative positions and, to some extent, the structural requirements for ligand interactions at each position. αY93, αY190 and γW55 are important for the binding of dTC, whereas the contributions of αY198 to the formation of the dTC site appear to be minor (O'Leary and White, 1992; O'Leary *et al.*, 1994). In the mouse, the relative importance of the contributions of αY93, αY190, and αY198 was demonstrated for acetylcholine and other agonists such as carbamoylcholine and suberyldicholine, although the rank order of importance of each residue was dependent on the ligand (O'Leary *et al.*, 1994; Sine *et al.*, 1994; Aylwin and White, 1994). The contributions of amino acids present in non-α subunits have also been studied. The role of Y111 of the γ-subunit as conferring high affinity for antagonists was delineated in a mutagenesis study in which the homologous arginine (R) residue on the δ subunit (which confers low affinity for the same compounds) was replaced by tyrosine (R113Y), the result of which was a 1000-fold increase in equilibrium binding affinity for various snake toxins (Chiara *et al.*, 1999). Y117 of the γ subunit of the mouse nAChR also confers affinity for the antagonist dimethyl-*d*-tubocurarine (DMT) (Fu and Sine, 1994). Another amino acid residue of the γ subunit that has now been shown to be involved in the binding of acetylcholine in the desensitized state is E57 (Prince and Sine, 1996). Further, other residues within the γ subunits have been implicated in conferring selectivity for DMT affinity at the α-γ/δ interfaces of mouse nAChR, including γI116, γY117, and γS161 (Bren and Sine, 1997). Residues in the homologous positions in the δ subunit are V118, T119, and K163 (Bren and Sine, 1997; Sine *et al.*, 1995). The same group has also determined residues of the γ and δ subunits which confer affinity to α-conotoxin M-1 and carbamoylcholine, including the following: γK34, γF172, γC115, δY117, δI178, and δS36 (Sine *et al.*, 1995; Prince and Sine, 1996). However, the selectivity conferred by certain residues may, in fact, be species specific. For example, C115 of the mouse γ subunit is not conserved in all species and in *Torpedo* receptor is replaced by serine. The substituted cysteine accessibility method (SCAM) has now been applied to the binding site of nAChR which has revealed that only the following residues are accessible to acetylcholine, by virtue of their modification by methanethiosulfonate (MTS) derivatives: αY93, αY198, αN94, and γE57 (Sullivan and Cohen, 2000). Interestingly, although γW55 and δW57 have been implicated in the formation of the acetylcholine binding sites by photolabeling and mutagenesis studies, these residues were not accessible to modification.

Although the aromatic amino acid residues of the α,γ, and δ subunits are reputed to from a negative anionic subsite within the acetylcholine-binding domain by virtue of cation-π bonding (O'Leary *et al.*, 1994), the contributions of aspartate and glutamate residues were evaluated using S-(2-[^{3}H]glycylamidoethyl)dithio-2-pyridine, a cross-linking agent that reacts specifically with sulfhydryl and carboxyl groups (Czajkowski and Karlin, 1991). This compound limits the distance of reacting residues, but not the direction, to 0.9 nm (Martin *et al.*, 1996). This approach revealed that the reactive C192/C193 disulfide bond could be cross-linked to an aspartate (D) /glutamate (E) rich region of the δ subunit between residues 164 and 224 (Czajkowski and Karlin, 1991). Systematic mutation of all aspartate and glutamate residues within this region to asparagine (N) and glutamine (Q), respectively, revealed the importance of δD180 and δE189, which, when mutated, reduced the affinity of acetylcholine by 1000 and 10-fold, respectively (Czajkowski and Karlin, 1993). Peptide mapping subsequently showed that δD165, δD180 and δE182 could all react with S-(2-[^{3}H]glycylamidoethyl)dithio-2-pyridine; these data, when combined with the mutagenesis data from the same group, illustrated the role of δD180 in the partial formation of a negative subsite within the acetylcholine recognition pocket (Czajkowski and Karlin, 1995). Mutation of the homologue to δD180, γD174, later confirmed that this negatively charged residue, in addition to γE183, may be necessary for agonist binding at the α-γ interface (Martin *et al.*, 1996).

These studies of nAChR have provided important information as to domains of a given subunit that are required for the formation of a ligand recognition site. There are several emergent themes from these data. The binding sites of agonists and competitive antagonists are not confined to a single subunit and residues involved in binding are located in non-contiguous domains of the primary sequence of a subunit (Fig. 1-1B). The labeling of amino acids at the α–γ or α–δ interfaces occurs primarily at aromatic residues that are located in disparate regions of the α, γ, and δ, subunits. Initially proposed by Galzi *et al.* (1990), the "loops" model of ligand recognition has been widely adopted for all receptors within the structurally homologous family of LGIC. The initial photolabeling of residues in the α subunit revealed that labelled residues were found in discrete clusters of the primary protein sequence of each subunit; in accordance with their regional location, the arbitrary convention of loops A-C was applied to the α subunit to describe the relative positions of the domains that had been identified to date. Specifically, W86 and Y93 are found within "loop A", while W149 and Y151 fall within "loop B". Amino acid residues within "loop C" of the α subunit include Y190, C192, C193, and Y198. This model has been further extended to account for amino acid residues of non-α subunits, such that the conserved tryptophan residues of the γ (W55) and δ (W57) subunits lie in "loop D". To date, amino acid residues of other loops have been identified, which have been designated "loops E" (Y111/Y117 of γ subunit) and "F" (D165, D180, E182, and E189 of the δ subunit and D174 and E183 of the γ subunit) (Fig. 1-2). This nomenclature is used to identify regions of adjacent subunits that form a single binding site. Since all subunits share about 40% sequence identity, it is possible that each carries domains that contribute to two different binding sites (i.e. loops of the same subunit can potentially form recognition sites at the two interfaces that any given subunit will form with its adjacent partners within the pentameric protein structure). Although this model has some shortcomings that do not account for the spatial orientation of the loops in the tertiary structure of the protein, the orientation of the ligand within the binding pockets, the relative distance between loops, the structural requirements for ligand interactions or the accessibility of these residues to neurotransmitters and their antagonists, it provides the conceptual framework by which to make predictions about binding site determinants for all receptors within this superfamily (Figs. 1-3, 1-4). Finally, the last emergent theme with respect to ligand recognition properties of this receptor is the importance of aromaticity. Thus, tyrosine and tryptophan residues appear to form a negative anionic subsite that could potentially stabilize the interaction with the positively charged ammonium group of acetylcholine (Galzi *et al.*, 1990).

GLYCINE AND 5HT$_3$ RECEPTORS

Glycine receptors are pentameric protein complexes composed of α(1-4) and β subunits in a 3α: 2β stoichiometry (Kuhse *et al.*, 1993; Griffon *et al.*, 1999). Chemical modification studies first suggested that the binding of the competitive antagonist, strychnine, was altered by modification of tyrosine, lysine or arginine residues (Ruiz Gomez *et al.*, 1990). The initial biochemical approaches in which photolabeling and chemical modification were combined with peptide mapping protocols localized the strychnine binding domain to residues 171-220 of the α subunit (Ruiz-Gomez *et al.*, 1990). Subsequent mutagenesis studies confirmed the participation of F159, G160 and Y161 ("loop B") of the α1 subunit, in addition to K200 and Y202 of the α subunit ("loop C") as forming part of the strychnine binding site (Kuhse *et al.*, 1990; Vandenberg *et al.*, 1992a). D148 is also reported to contribute to the binding of strychnine, but this residue is equally important for receptor assembly (Vandenberg *et al.*, 1993). As in the nAChR, mutagenesis studies of the GlyR have revealed that the determinants of agonist and antagonist binding need not overlap, although they appear to be located in the same general regions of the protein. This was first shown when T204 ("loop C") produced alterations in the apparent affinity of glycine, while that of strychnine was unaffected (Vandenberg *et al.*, 1992b). The same group next demonstrated that, in fact, K200, Y202 and T204 were important for agonist binding (Rajendra *et al.*, 1995). Finally, the most recent data on the binding of glycinergic ligands suggest that several residues in the α1 subunit contribute to the apparent affinity for glycine, but do not impair the ability of strychnine to antagonize the response to the agonist (Vafa *et al.*, 1999). These residues, I91, A101, N102 and possibly also W94 are all found within "loop A" (Vafa *et al.*, 1999). Further, determinants of antagonist efficacies within the same domain of the α subunit have now been established, and these include the following: K104, F108, and T112 (Schmieden *et al.*, 1999).

The $5HT_{3A}R$ remains the least well-characterized receptor of this family, although the recent cloning of a novel subunit (B) (Davies *et al.*, 1999) will undoubtedly fuel further biochemical and electrophysiological characterization of the binding sites on these receptors. To date, however, the information that has been reported suggests that the model that describes the binding sites of the other receptors (both anionic and cationic) is also applicable to the apparently homomeric $5HT_{3A}$ receptor that has been used in those mutagenesis studies so far. The first report of mutagenesis described the importance of E106 ("loop A") in which it was hypothesized that this residue could potentially form a hydrogen or ionic bind with the primary ammonium group of the agonist, 5-HT (Boess *et al.*, 1997). A subsequent study by the same investigators described the importance of the adjacent phenylalanine (F107) to the recognition properties of the receptors, although mutation to tyrosine or asparagine residues produced relatively small changes in the affinity of most antagonists (Steward *et al.*, 2000). Other studies of the $5HT_{3A}R$ have suggested that W89 is important for the binding affinities of curare and granisetron (Yan *et al.*, 1992), while R91 is an important determinant of 5HT affinity. These authors have also shown that this region ("loop D") appears to from a β-strand because every second residue (W89, R91, and F93) differentially alters the affinity for the competitive antagonist, granisetron (Yan *et al.*, 1992). This suggests that the structural features of "loop D" of these receptors may be conserved throughout the LGIC family since "loop D" of the α1 subunit of $GABA_AR$ has been shown by SCAM to form a β-strand (Boileau and Czajkowski, 1999). Finally, mutagenesis has clarified the contribution of tryptophan residues to ligand recognition such that W90, W183, and W195 have all been implicated in ligand recognition in $5HT_{3A}R$ (Spier and Lummis, 2000). W90 ("loop D") is homologous to F64 ($GABA_AR$ α1), W183 is homologous to W149 ("loop B") (nAChR a subunit), while W195 appears to represent a completely novel domain for agonist binding to this class of receptors (Spier and Lummis, 2000). It has been postulated that these tryptophan residues form part of a cation-π stacking complex that mediates, in part, the binding of the serotonergic ligands.

SUBUNIT INTERFACES FORM BINDING SITES FOR $GABA_AR$ AGONISTS AND BENZODIAZEPINE LIGANDS

Photolabeling and peptide mapping experiments have elucidated important information as to the subunits required for the binding of [^{3}H]flunitrazepam, [^{3}H]Ro15-4513, and [^{3}H]muscimol. The first of these studies suggested that [^{3}H]flunitrazepam and [^{3}H]muscimol were incorporated into the α and β subunits, respectively (Casalotti *et al.*, 1986), a finding that was confirmed independently by Deng *et al.* (1986). Further investigations suggested that, in addition to its labeling of the β subunit, muscimol could, in some cases, label an α subunit. Though minor in comparison to the β subunit labeling, the incorporation into the α subunit reached a maximum of 38% of the total β subunit labeling in human brain membranes (Bureau and Olsen, 1988). This was perhaps some of the earliest data to suggest that the binding site for [^{3}H]muscimol occurred at the interface between α and β subunits. A series of studies from the same laboratory followed in which [^{3}H]muscimol was reported to label a heterogeneous population of β subunits and also the α subunit, although this was not readily resolved on an acrylamide gel (Bureau and Olsen, 1990; Bureau and Olsen, 1993). In fact, a weak labeling of F64 of the α1 subunit was reported in the following year, but this remains controversial (Smith and Olsen, 1994), because (1) this site is likely to be involved in a low affinity binding (see below) and (2) [^{3}H]muscimol can only reproducibly photolabel high affinity binding sites, especially as the efficiency of incorporation (~7%) of [^{3}H]muscimol is poor (Casalotti *et al.*, 1986).

[^{3}H]Flunitrazepam and [^{3}H]Ro15-4513 have proved to be useful photolabels in identifying domains of the $GABA_AR$ that contribute to the formation of a benzodiazepine binding site. Early peptide mapping studies with [^{3}H]flunitrazepam suggested that the primary site of photoincorporation was likely found in the domain between residues 58-149 of the α subunit (Stephenson and Duggan, 1989). It was also demonstrated that in addition to the α subunit, the γ2 subunit was also an integral component of the photolabeling reaction (Stephenson *et al.*, 1990). Therefore, photolabeling data has provided information that the binding site for the benzodiazepines is located at the interface between adjacent α and γ subunits. The localization of these labels was subsequently refined when it was demonstrated that H102 of the bovine GABA$_A$R was the major site of [^{3}H]flunitrazepam photoincorporation (Duncalfe *et al.*, 1996). Mapping studies also confirmed the differential labeling of the α subunit by flunitrazepam and Ro15-4513 (Davies *et al.*, 1996; Duncalfe *et al.*, 1995). Specifically, hydroxylamine cleavage patterns suggested that the localization of the flunitrazepam label occurred N-terminal to residue 103, while Ro15-4513 labelled a domain that occurred between residues 104 and the C-terminus (Davies *et al.*, 1996). An additional residue of the bovine α1 subunit, P97, has recently been identified by photolabeling and peptide mapping as being a contact residue for [^{3}H]flunitrazepam (Smith and Olsen, 2000). Finally, Davies and Dunn (1998) have reported that the partial inverse agonist, Ro15-4513, recognizes a unique domain of the GABA$_A$R that, on the basis of peptide mapping studies, has been localized to the domain that includes residues 247-281 of the α1 subunit.

BENZODIAZEPINE SITE: THE α–γ INTERFACE

Of the allosteric modulators of GABA$_A$R function, the benzodiazepines have been the most widely studied, due, in part, to the availability of suitable radioligands for direct investigations of putative binding sites, and because they are of high affinity and have low non-specific interactions. Early photolabeling data suggested that the α subunit was the primary site of photoincorporation of [^{3}H]flunitrazepam (Casalotti *et al.*, 1986; Deng *et al.*, 1986), but that there was at least minor labeling of the γ2 subunit (Stephenson *et al.*, 1990). This information suggested that, like agonists, the benzodiazepine site of GABA$_A$R was formed at the interface between adjacent α and γ subunits (Fig. 1-5) (Stephenson *et al.*, 1990), which corroborated the fact that the γ subunit is required for benzodiazepine pharmacology (Pritchett *et al.*, 1989a). Photolabeling studies later demonstrated that the major site of incorporation of [^{3}H]flunitrazepam is H101 (Duncalfe *et al.*, 1995; Smith and Olsen, 2000), although residual labeling of P96 has been recently reported (Smith and Olsen, 2000). To date, no other residues have been implicated by photolabeling. Mutagenesis, however, has proved to be a very powerful methodology in defining structural elements for binding. Among the α subunits, only α4 and α6 give rise to receptors that are insensitive to classical benzodiazepines such as diazepam and flunitrazepam, but they recognize the partial inverse agonist, Ro15-4513, with high affinity (Scholze *et al.*, 1994; Hadingham *et al.*, 1996). The α6 subunit was therefore used in conjunction with the diazepam-sensitive α1 subunits in a study to determine which residues were important for diazepam recognition. The generation of chimaeric proteins and subsequent mutagenesis identified H101 of the α1 subunit ("loop A") as the residue that confers high affinity to diazepam and Ro15-1788 (Wieland *et al.*, 1992; Davies *et al.*, 1998a) in addition to diazepam potentiation of GABA-mediated chloride conductance (Kleingoor *et al.*, 1993). In α4 and α6 subunits, the residue in the homologous position is arginine. Subsequent to the identification of H101, Wieland and Lüddens (1994) generated an additional series of chimaeras and mutants, the aim of which was to restore α1-like affinity to α6(R100H)β2γ2 receptors. In fact, an additional study identified amino acid residues (in α1: T162, G200, and V211) that are important for the affinity of agonists, but not Ro15-4513 (Wieland and Lüddens, 1994). Further, T162 ("loop" B), G200, and S204 ("loop" C) are important for zolpidem binding (*see* also Pritchett and Seeburg, 1991; Schaerer *et al.*, 1998). Based on homology considerations with the (low affinity) GABA binding site, other elements required for benzodiazepine potentiation of GABA-mediated current have been identified; specifically, these include αY161 ("loop B"), αT206 ("loop C"), and γF77 ("loop D") (Buhr *et al.*, 1996). Subsequent studies illustrated the importance of αY159 ("loop B") (Amin *et al.*, 1997), αT206 (Buhr *et al.*, 1997a), αY209 ("loop C") (Buhr *et al.*, 1997a: Amin *et al.*, 1997) and γF77 (Buhr *et al.*, 1997b; Wingrove *et al.*, 1997). An additional residue of the γ subunit (M130) has been identified as a key determinant of the benzodiazepine binding site ("loop" E) (Wingrove *et al.*, 1997). More recently, the use of substituted cysteine accessibility methodology (SCAM) to study the γ2 subunit has revealed that additional residues including T73, D75, A79, T81 and Y83 are accessible to modification by MTSEA-Biotin (Teissére *et al.*, 1999) and, of these, A79 plays a specific role in the formation of the binding pocket because, unlike other residues within this domain, A79 is protected from modification by the agonist, flurazepam. In addition to A79, it has further been shown that M57/T58 and ("loop" D) are important determinants of benzodiazepine binding in the γ2 subunit (Kucken *et al.*, 2000). Another mutagenesis study suggests that T142 ("loop" E) of the γ2 subunit (Mihic *et al.*, 1993) is important for determining benzodiazepine efficacy.

LOW AFFINITY GABA RECOGNITION SITE: β–α INTERFACES

Studies of $GABA_AR$ have revealed that neurotransmitter binding sites are located at the interfaces of adjacent subunits and that amino acids important for binding lie in structurally homologous positions to the prototypical nAChR (Figs. 1-3, 1-4). However, unlike the benzodiazepine site, relatively little information about the channel activation sites of $GABA_AR$ is available. The first structural information came from a discrepancy in published amino acid sequences of the α1 subunit. Malherbe *et al.* (1990) had published a sequence of α1 that differed from another reported sequence (Khrestchtisky *et al.*, 1989) in that position 64 was reported to be occupied by leucine though others had reported a phenylalanine at the same position. Sigel *et al.* (1992) subsequently altered the primary sequence of their α1 subunit protein by substitution of phenylalanine at this position and the resulting data implicated F64 ("loop D") in channel activation since expression of F64L produced a 200-fold rightward shift in concentration-response curves; this mutation also altered the apparent affinity of the competitive antagonist, bicuculline methochloride. The effects of this substitution at homologous positions in the β2 and γ2 subunits had only minor effects on the apparent affinity of GABA. More recently, the use of SCAM techniques to probe the area around F64 has suggested that two additional amino acid residues (R66 and S68) are accessible to modification by MTSEA-biotin and line part of the binding domain in such as way that the secondary structure of this region is likely to be a β-strand (Boileau and Czajkowski, 1999). The channel activation site is not restricted to residues on the α subunit. Two homologous domains of the β subunit that were required for channel gating by both GABA and muscimol (Amin and Weiss, 1993) have been identified. In that paper, the residues Y157, T160 ("loop B"), T202, and Y205 ("loop C") were shown to shift the concentration-effect relationship to the right without altering the maximum amplitude of the current. In this respect, the authors attribute these effects to ligand binding rather than channel gating. Other residues of the α subunit that play a role in this site include R120 (Westh-Hansen *et al.*, 1999) and I121 (Westh-Hansen *et al.*, 1997), both of which are found in a region that is homologous to loop E of the nAChR. To date, no residues have been implicated in "loop" A (Fig. 1-6).

OTHER BINDING SITES OF GABA$_A$R

In addition to the agonist and benzodiazepine sites, there are a number of allosteric sites that have been less well characterized than the former, due in part to (1) the absence of suitable radioligands for the direct investigation and (2) their low affinity. In these cases, effects of these modulators, direct or allosteric, have been investigated using electrophysiological techniques or biochemical means. In the former, it is possible to measure the modulating effects of a variety of agents and their direct effects, whereas in using reporter [^{3}H]ligands, it is only possible to measure the ability of these compounds to affect the binding of agonists or benzodiazepine ligands. Therefore, the barbiturates, divalent cations, neurosteroids and metabolites of endogenous steroids, general anaesthetics and alcohol, have all been less well characterized in terms of the structural basis for ligand binding than benzodiazepine compounds because most, with the exception of *tert*-butylbicyclophosphorothionate (TBPS), are low affinity ligands.

The barbiturates, propofol, etomidate, and certain neurosteroids are generally grouped together because they are all anesthetics, although each has a distinct chemical structure. The effects that each class of compound has on GABA$_A$R function are qualitatively similar, though their mechanisms of action appear to differ. Unlike benzodiazepines, it appears that there are less rigid subunit requirements in order to observe the effects of these agents. Specifically, unlike the benzodiazepines the barbiturates exert their effects even in the presence of either the α4 or α6 subunit (Wafford *et al.*, 1996). The neurosteroids, however, appear to interact preferentially with receptors in which the α1, β1 and γ2 subunits are assembled (Puia *et al.*, 1990). However, unlike benzodiazepines, neurosteroids and barbiturates do not require the presence of a γ subunit for their overt effects (Schofield *et al.*, 1987; Puia *et al.*, 1990). Divalent cations, such as zinc ions, can block the functional response to GABA in which α and β subunits are expressed; this block, however, is alleviated by the expression of a γ subunit (Smart *et al.*, 1991). The effects of ethanol, on the other hand, have been more difficult to study and the findings remains somewhat controversial; however, it does seem to alter the response to GABA at α1β2γ2 receptors (Ueno *et al.*, 1999). Finally, the channel blocker, picrotoxin, is effective on heterologous expression of β3, α1β3, α1β2, α1β3γ2, α1β2γ2 and β3γ2 subunits, but not on expressed α1γ2, α1, or γ2 receptors alone (Pregenzer *et al.*, 1993; Zezula *et al.*, 1996). Therefore, like the benzodiazepines, other compounds that modulate $GABA_AR$ function show subunit dependence to some degree. Other compounds that are reported to alter GABA-gated chloride conductance include melatonin, trichloroethanol, chlormethiazole, lanthanum, spermine, and spermidine (*see* Sieghart, 1995). The subunit dependence of these compounds has not been extensively investigated.

Pentobarbital, propofol, etomidate, and the neurosteroid, 5α-pregnan-3α-ol-20-one, all appear to potentiate GABA-gated chloride flux at low concentrations, but directly gate the channel at high concentrations (for review, *see* Belleli *et al.*, 1999). The sites of action that mediate these two effects appear to be different since a point mutation within TMII (N289M) of the β3 subunit reduces the GABA-mimetic effects of propofol (Belleli *et al.*, 1999). Further, Y157 of the β2 subunit has been shown to be important in conferring affinity for propofol (Fukami *et al.*, 1999). Propofol has also been reported to increase the affinity of low affinity binding sites (Davies *et al.*, 1998b) and to potentiate the binding of [^{3}H]flunitrazepam, while inhibiting the binding of [^{3}H]Ro15-4513. Recent studies have suggested that the mode of action of propofol is to increase the efficacy of agonists at recombinant α1β2γ2 and α1β2 $GABA_AR$ (O'Shea and Harrison, 2000). Finally, Lam and Reynolds (1998) have demonstrated that the efficacy of propofol at α1β2 receptors is greater than at α1β2γ2 receptors, the implication of which is that expression of a γ subunit reduces that ability of propofol to directly activate the channel. This, the authors, speculate may suggest that there are multiple binding sites for propofol, which mediate, in part, its ability to modulate and gate the channel in the presence or absence of the endogenous neurotransmitter.

No specific high affinity binding sites for barbiturates have been identified. Pentobarbital increases the affinity of low affinity [^{3}H]muscimol binding sites, and potentiates the binding of [^{3}H]flunitrazepam, while, at the same time, decreases the binding of [^{3}H]Ro15-4513 (Davies *et al.*, 1998b). However, while mutational analysis has been limited, the N289M substitution of the β3 subunit does not alter the modulatory effects of pentobarbital, but does reduce its GABA-mimetic effects. Similarly, the same mutation impaired both the modulatory actions of etomidate and its direct effects without influencing either of the effects of the neurosteroid (Belleli *et al.*, 1999). This suggests that the mutation is specific for the intravenous anaesthetic, which might therefore indicate specificity for the binding of propofol or interruption of the transduction machinery for both propofol and etomidate (Belleli *et al.*, 1999). However, without the use of radioligands to investigate these binding sites directly, this conclusion remains speculative. Other residues, which have been mutated, that do not affect the ability of pentobarbital to elicit its effects are Y157, T161, T202, and Y205 (Amin and Weiss, 1993), which suggests that the GABA and barbiturate sites are distinct. The mutation Y157F likewise did not alter the direct functional responses of etomidate, pentobarbital or the modulatory responses of etomidate, pentobarbital, or propofol (Fukami *et al.*, 1999). However, Belleli *et al.*, (1999) demonstrated that the mutation I307S of the ρ1 subunit prevented direct activation of this receptor subtype by pentobarbital, whereas the T262Q mutation of the β1 subunit prevented the modulatory effects of pentobarbital, but did not prevent the receptor from being directly activated (Dalziel *et al.*, 1999). Clearly, these early studies represent an important point of departure for the study of the mechanisms of action of general anaesthetics and will undoubtedly form the basis for a complex study of these compounds.

Residues that have been implicated in altering the potentiation of GABA current by ethanol include S270 and A291 of the α subunit, in addition to S265 and S280 of the β1 subunit (Ueno *et al.*, 1999). From these studies, it is known, at least in the studies to date, that residues in the α and β subunits are more important than residues in the γ subunit for conferring the potentiating effects of ethanol (Ueno *et al.*, 1999). The study of the inhibition of current by zinc ions has elucidated the roles of several amino acids. Specifically, it has been demonstrated that H273 of α6, H267 of β1 and H292 of β3 are important in conferring zinc ion sensitivity (Hornstein and Akabas, 1998). Finally, residues that are important in conferring TBPS/picrotoxin affinity include A252 and L253 of the β3 subunit, both of which are located in the second transmembrane domain (Jursky *et al.*, 2000).

HETEROGENEITY OF EQUILIBRIUM BINDING: AGONISTS/ANTAGONISTS

Equilibrium binding studies have revealed the presence of multiple classes of agonist sites, which differ in their affinity by at least an order of magnitude for GABA or its conformationally restricted analogue, muscimol. This apparent heterogeneity is present in membrane preparations from a number of species, including rat (Enna and Snyder, 1977; Beaumont *et al.*, 1978; Greenlee *et al.*, 1978a; Guidotti *et al.*, 1979; Williams and Risley, 1979; Guidotti *et al.*, 1981; Kurioka *et al.*, 1981a; Kurioka *et al.*, 1981b; Olsen *et al.*, 1981; Matsumodo and Fukuda, 1982; Skerritt and Johnston, 1983a; Concas *et al.*, 1985; Corda *et al.*, 1986; Corda *et al.*, 1987; Shank *et al.*, 1990), mouse (Wang *et al.*, 1979; Yang and Olsen, 1988), cow (Olsen *et al.*, 1981; Agey and Dunn, 1989; Costa *et al.*, 1990), bovine retina (Madtes Jr., 1984; Madtes Jr. *et al.*, 1989), chicken (Fiszer de Plazas *et al.*, 1993) and recombinant receptors expressed in either Ltk- cells (Davies *et al.*, 1994) or tsA201 cells (Newell *et al.*, 2000). Generally, it is accepted that there are at least two classes of binding sites, the parameters of which can be measured using radioligand binding techniques. However, some investigators have suggested that it is likely that there is a third class of 'ultralow' affinity (>1 μM) sites that mediate ion channel opening (Olsen *et al.*, 1986). This is based on the fact that concentrations of agonist required to elicit current are one to three orders of magnitude greater than the apparent dissociation constants of both high (K_D = 10–30 nM) and low affinity sites (K_D = 0.1–1.0 μM). These two binding affinities are measured in most receptor preparations, irrespective of the brain region from which the membranes are derived. However, it does appear that the maximal binding density is higher in cortex and cerebellum than other areas (Guidotti *et al.*, 1979) and that there can be different proportions of high and low affinity sites in specific brain regions such that the cerebellum has a greater proportion of high affinity sites (Olsen *et al.*, 1981). Further, the number of agonist binding sites on a single receptor has not been clearly defined (Hevers and Lüddens, 1998). It is generally accepted that the stoichiometry of high affinity muscimol binding sites to benzodiazepine binding sites is 2:1 (Sigel *et al.*, 1983; Sigel and Barnard, 1984; Stephenson, 1988; Farrar *et al.*, 1999), which, in the simplest case, could reflect the fact that two molecules of agonist are required for channel opening (Sigel and Buhr, 1997). However, the channel activation sites as measured in electrophysiological studies are likely of low affinity and not readily detectable in radioligand binding assays. Nonetheless, in support of the studies listed above it is also generally presumed that there are at least two channel activation sites because Hill coefficients >1 imply that there are multiple binding sites. Thus, since high and low affinity sites exist under equilibrium conditions, a single receptor could carry at least four binding sites for GABA. Finally, the high affinity benzodiazepine site may represent a (very) low affinity agonist binding site, which throughout the course of evolution, has become a recognition domain for these therapeutic compounds (Boileau and Czajkowski, 1999). Therefore, a single receptor could potentially carry up to five agonist binding sites, all of which may occur at unique subunit-subunit interfaces.

The absolute K_D values for these sites vary depending on the membrane preparations (i.e. fresh or frozen), choice of buffers, assay temperature, and the method used to separate bound and free ligand (Table 1-1). Assay conditions to measure binding parameters of [^{3}H]agonist have been studied extensively and there is no clear consensus as to the optimal parameters for measuring the binding of these ligands. Although centrifugation and filtration techniques have been used to separate bound and free ligand in studies of this nature, filtration appears to yield a higher ratio of specific to non-specific binding and is less variable than centrifugation methods (Shank *et al.*, 1990). The introduction of rapid filtration techniques permits even greater resolution of low affinity binding sites (Agey and Dunn, 1989). Most binding assays are carried out a 4°C (Olsen *et al.*, 1981; Agey and Dunn, 1989; Newell *et al.*, 2000), although high affinity muscimol binding can be measured equally well at 25°C or 37°C (Shank *et al.*, 1990). Other investigators suggest that incubation and filtration at 22°C decreases the affinity of high affinity sites and does not permit the resolution of multiple classes of binding sites

(Yang and Olsen, 1987; Boileau and Czajkowski, 1999). The optimum pH for these binding assays falls in the range of 7.1-8.0, and the specific binding of [^{3}H]muscimol or [^{3}H]GABA decreases below and above this range (Enna and Snyder, 1975; Olsen *et al.*, 1981; Madtes Jr., 1984). Specific binding appears to be increased by repeated washing of brain membranes (Shank *et al.*, 1990) and multiple freeze-thaw cycles (Enna and Snyder, 1975; Beaumont *et al.*, 1978;), although other researchers suggest that specific binding is higher in freshly prepared membranes (Shank *et al.*, 1990). Exhaustive washing is, however, necessary to quantitate accurately the density of low affinity binding sites accurately (Fiszer de Plazas, 1993). The inclusion of detergents in the preparation of membranes appears to be an additional factor that has further complicated this field. The addition of Triton-X-100 reveals an additional class of high affinity [^{3}H]GABA binding sites (Guidotti *et al.*, 1979), but addition of Triton-X-100 (0.1% w/v) or Lubrol PX (0.1% w/v) does not appear to affect the binding of [^{3}H]muscimol (Wang *et al.*, 1979), although some investigators have reported a potentiation of [^{3}H]muscimol binding on the order of ~2.5-fold when these detergents are used (Williams and Risley, 1979). Higher concentrations of this detergent, on the other hand, decrease [^{3}H]muscimol binding (Guidotti *et al.*, 1979). The choice of buffer is said to alter the specific binding of GABA and muscimol in that physiological buffers (e.g. Krebs) increase the B_{max} for GABA, whereas the B_{max} is greater for muscimol in non-physiological buffers (e.g. Tris-HCl, Tris-citrate). K_D values for muscimol show only a slight dependence on the composition of the buffer, with the absolute values being slightly higher in Tris buffers (Madtes Jr. *et al.*, 1989). Finally, the inclusion of sodium ions in assay buffers appears to alter the binding of [^{3}H]GABA, but has no effect on the binding of [^{3}H]muscimol (Kurioka *et al.*, 1981). Some have suggested that the binding affinities are higher in the presence of sodium because it is thought to reflect the binding to transport sites instead of receptor sites, such that sodium-ion free buffers have largely been adopted in the radioligand binding assays. In the presence of sodium chloride, the binding of GABA increases in a concentration dependent fashion, but decreases beyond 200mM sodium chloride (Enna and Snyder, 1977; Olsen *et al.*, 1981; Shank *et al.*, 1990). Apparent synergistic effects of buffers, detergents, and sodium chloride further complicate the choice of buffer. It has been reported that the inclusion of Triton-X-100 and sodium ions in Tris-sulfate buffers (pH 7.4) permits the resolution of one class of sites, whereas the exclusion of sodium ions from the same buffer allows for the resolution of two classes of binding sites (Kurioka *et al.*, 1981a, b).

Despite the apparent lack of agreement as to the optimal conditions for the measurement of agonist binding, most methods appear to produce Scatchard plots that have multiple components. The existence of curvilinear Scatchard plots from brain membrane preparations suggests many potential mechanisms for agonist binding. These include the following: (1) multiple classes of independent sites, (2) interconvertible states of a single binding site, (3) negative cooperativity, or (4) multiple receptor subtypes in brain membrane preparations (Greenlee *et al.*, 1978b; Agey and Dunn, 1989). On the basis of equilibrium binding studies alone, it is not possible to discriminate among these mechanisms. Guidotti and coworkers (1979) first suggested that high and low affinity binding sites are distinct on the basis of the uneven distribution of high and low affinity binding in certain brain regions and that only the high affinity binding sites were regulated by an as yet unidentified endogenous protein modulator. Heterogeneity in equilibrium binding [for which the receptor classification of $GABA_1$ and $GABA_2$ was coined (Enna and Snyder, 1975)] was originally thought to reflect the existence of multiple $GABA_A$ receptor subtypes, which differed in their affinity for GABA in the brain (Enna and Snyder, 1975). Later studies suggested that heterogeneity in ligand binding could be described by models of independent sites or multiple interconvertible states, although the latter was no more valid than the former as a description of the data and would serve to describe the same data set with equivalent validity when interconversion of binding sites does not occur (Olsen *et al.*, 1981). However, these authors argue that the sites are likely independent on the basis of complex association and dissociation kinetics in which multiple components are observed, irrespective of ligand concentration (Olsen *et al.*, 1981; Yang and Olsen, 1987; Agey and Dunn, 1989). This is further corroborated by the fact that heat inactivation studies selectively reduced the density of low affinity sites, but not high affinity sites (Olsen *et al.*, 1981). Chemical modification studies also showed that it is possible to modify either high or low affinity binding without altering the complementary component, the implication of which is that high and low affinity sites are independent (Burch *et al.*, 1982; Maksay and Ticku, 1984a; Skerritt and Johnson, 1982). Specifically, ρ-diazoniumbenzenesulfonic acid disrupts low affinity GABA binding, while ammonium thiocyanate selectively disrupts high affinity binding (Maksay and Ticku, 1984b). Further evidence for selective disruption comes from the fact that diazotized sulfanilate and tetranitromethane impair the ability of muscimol to potentiate [^{3}H]diazepam binding (Burch *et al.*, 1982; Maksay and Ticku, 1984a), while *N*-acetylimidazole impairs muscimol binding (Maksay and Ticku, 1984a). Additional evidence from biphasic binding of the competitive antagonists, bicuculline and SR95531, which recognize the agonist sites with an affinity that is inversely related to the agonist affinity (Maksay, 1988; Maksay 1994) suggest that there are two classes of antagonist sites (Möhler and Okada, 1978; Olsen and Snowman, 1982; Heaulme *et al.*, 1987). However, despite the availability of complex kinetic data, multiple agonist affinities are still explicable if multiple receptor subtypes are considered. The unequivocal demonstration that single receptor subtype carried two classes of binding sites of very high and low nanomolar affinity came when stable expression of the $\alpha 1\beta 2\gamma 2_S$ subtype was used in [^{3}H]muscimol binding assays (Davies *et al.*, 1994). These observations have also been validated by a recent mutagenesis study in which a novel determinant of high affinity binding was described (Newell *et al.*, 2000).

FUNCTIONAL ROLES OF LOW AND HIGH AFFINITY AGONIST BINDING SITES OF GABA$_A$R

The relative contributions of both high and low affinity binding sites to GABA$_A$R function are not clear. Following the identification of high and low affinity sites in brain membrane preparations, it was demonstrated that micromolar concentrations of GABA or muscimol could potentiate specific [^{3}H]diazepam binding in a concentration-dependent fashion (Tallman *et al.*, 1978). This interaction between GABA and the benzodiazepine site appears to be well correlated with the low affinity GABA sites since the half-maximal concentration for diazepam potentiation (1.6 μM, Tallman *et al.*, 1978) corresponded closely to the K_D value of low affinity binding sites (1.2 μM) obtained in saturation assays (Enna and Snyder, 1977). Chemical alterations of high affinity [^{3}H]muscimol binding did not affect the ability of GABA to potentiate specific [^{3}H]diazepam binding (Marangos and Martino, 1981). Other approaches such as chronic diazepam treatment modified the low affinity binding component by increasing its affinity, whereas the affinity and density of the high affinity sites were unchanged (Gallager *et al.*, 1984). Direct demonstrations of the modification of low affinity binding parameters has suggested that benzodiazepine positive allosteric modulators increase the K_D of low affinity sites without alterations to its B_{max} values or any of the high affinity binding site parameters (Skerritt *et al.* 1982a, b; Matsumoto and Fukuda, 1982; Skerritt and Johnston, 1983a, b; Páldi-Harris *et al.*, 1985; Bristow *et al.*, 1990). Generally, diazepam or ligands which share the same pharmacological profile potentiate GABA binding by approximately 40-45% (Skerritt and Johnston, 1982a; Skerritt *et al.*, 1983a,b; Pàldi-Harris *et al.*, 1985), while the potentiation of [^{3}H]muscimol binding is only about 20% (Matsumodo and Fukuda, 1982; Skerritt *et al.*, 1983b). The half-maximal concentration of diazepam required to mediate these effects is in the high nanomolar range (~ 20 nM), which is in good agreement with direct measurements of the binding parameters of this ligand (Davies *et al.*, 2000). Another study, however, reported that rather than affect affinity, the effects of benzodiazepine ligands were to increase the abundance of low affinity binding sites (Corda *et al.*, 1986). Finally, electrophysiological studies have demonstrated that positive allosteric modulators shift the curve to the left, which indicated that benzodiazepines interact with the low affinity (i.e. channel activation) sites (Dunn *et al.*, 1999; Chang and Weiss, 1999; Davies *et al.*, 2000).

The role of high affinity sites is not clear since their binding parameters are not generally modified by diazepam. However, one study by Meiners and Salama (1985) in which [^{3}H]GABA (20 nM) was employed as the reporter molecule showed that diazepam and the partial benzodiazepine agonist CG29896 increased the binding by about 20-45% with an EC_{50} value of 30 nM. There are reports, too, that the effects of barbiturates may be mediated via high affinity binding sites since pentobarbital modestly increases the relative proportion of high affinity sites in brain preparations (Olsen and Yang, 1987). There remains speculation as to the contributions of high affinity binding sites to receptor function. Some groups maintain that rather than being an independent site the high affinity binding site simply reflects the desensitized state of low affinity binding sites (Ebert *et al.*, 1997; Sigel and Buhr, 1997), while others suggest that the role of the high affinity binding site is related to desensitization processes (Cash and Subbarao, 1987a, b).

The latter is in good agreement with an early model of nAChR receptor activation and desensitization in which the two general receptor processes are mediated by binding to low and high affinity sites, respectively (Dunn and Raftery, 1982; Dunn *et al.*, 1983).

MODELS OF RECEPTOR ACTIVATION AND DESENSITIZATION

The binding of agonists to a receptor and the subsequent allosteric conformational changes that accompany ligand binding describe the activation of $GABA_AR$ by agonists. Few details concerning ion channel opening have been resolved but evidence from the nAChR suggests that the binding of agonist to its receptor produces localized conformation changes which are gradually transduced by small rotations to structures in the membrane, which, in the end, alter the channel gate, thereby allowing the flow of ions along their concentration gradient (Unwin, 1995). At the present time, it is not certain how many molecules are required to activate a native receptor, but it is presumed that mono- and di-liganded receptors can reach the open channel state (Jones *et al.*, 1998).

Desensitization is believed to subserve two main purposes, the first of which is to terminate the cellular response to an individual stimulus, and the second of which is to cause a general reduction in responsiveness without terminating the stimulus. The process of desensitization has received a great deal of attention, especially in terms of the multiple components of desensitization. Generally, desensitization is described by two components, which are termed fast and slow. It is generally accepted that ligand-gated ion channels undergo a conformational transition from the active state to a desensitized state on the basis of homology considerations with the nAChR. Unwin (1995) was able to capture the resting receptor and the desensitized receptor bound to molecules of acetylcholine and, in doing so, showed that the relative positions of the subunits within the receptor were different in each state. Thus, quaternary structure mediates the initial state of desensitization, which is in good agreement with a study by Lu and Huang (1998) in which elements within the amino terminus and carboxyl terminus were responsible for mediating these processes.

The role of receptor phosphorylation in the desensitization process remains ambiguous. Several studies have examined the phosphorylation of $GABA_AR$, but this work remains controversial. PKC has been shown to mediate internalization of receptors

(Filipova *et al.*, 2000), but does not alter the desensitization kinetics of $GABA_AR$ (Lin *et al.*, 1996). Further, PKC has been shown to increase the maximum current amplitude in $\alpha1\beta1\gamma2$ receptors, which has been attributed to phosphorylation of serine residues 409 ($\beta2$), 237 ($\gamma2$) and 343 ($\gamma2$). However, other investigators report that PKC decreases the amplitude of GABA current (Sigel and Baur, 1988), which casts some doubt on the validity of these results. Likewise, phosphorylation of tyrosine residues 365 and 367 of the $\gamma2$ subunit has been shown to enhance GABA whole cell current (Moss *et al.*, 1992). However, PKA inhibits GABA currents and is reported to phosphorylate S409 of the $\beta1$ subunit. In that study, however, PKA was shown to decrease the proportion of the fast component of desensitization, while having little effect on the time course for each of these components. Finally, it has been demonstrated that the rate of desensitization is decreased in chick cortical neurons when cyclic adenosine monophosphate is applied to cells (Tehrani *et al.*, 1989).

Many models that account for multiple binding steps and the allosteric transition from the resting state to the active state have described the activation processes of $GABA_AR$. Generally, it is presumed that two molecules of agonist are required to activate the receptor since Hill coefficients greater than unity imply multiple binding steps. The simplest of these models, postulated by Del Castillo and Katz (1957), requires sequential binding of agonist molecules, which promote isomerization to the active state. This model, although an oversimplification of a complex process, does not account for multiple classes of binding sites on a single receptor macromolecule, and suggests that the same class of binding site mediates activation and desensitization. However, despite the apparent lack of complexity, this model serves as the basis by which most mutagenesis data is interpreted because it does not require detailed kinetic analysis to rationalize changes in ligand binding or channel gating, which, as described by Del Castillo and Katz (1957) could be a function of either the initial binding steps or the transition to the active state of the receptor. This model has also been extended to account for desensitization processes such that the linear scheme has been superseded by a cyclical model of receptor activation and desensitization (Fig. 1-7A).

However, this model assumes that ligand binding is a simple process, although the validity of this assumption has recently been challenged by a kinetic study of receptor affinity wherein an energy barrier was shown to limit the binding process (Jones *et al.*, 1998). Thus, small molecules that recognize the $GABA_AR$, which do not overcome a significant energy barrier must climb out of a deep energy well and thus unbind more slowly than lower affinity ligands which have significantly greater energy requirements for ligand binding (Jones *et al.*, 1998). This newer version of the model accounts for multiple phases of desensitization (e.g. fast and slow), which are predicted to occur from the doubly liganded state and the mono-liganded state of $GABA_AR$. Since recovery of receptors from desensitization is almost entirely dependent on dissociation of the GABA molecules from the receptor (Clements, 1996), this model can be interpreted in light of two classes of binding sites. Thus, the rapid phase of desensitization could be attributed to dissociation from low affinity sites, whereas the slower component could represent dissociation from high affinity binding sites (Fig. 1-7B).

One of the most widely accepted models for the transitions of allosteric proteins was put forth by Monod, Wyman, and Changeux (1965). This model describes the structure of the protein as being composed of a distinct number of subunits that occupy equivalent positions within the final receptor molecule. The basic tenet of this model is that proteins exist in two distinct conformational states, between which there is equilibrium in the absence of ligand. Further, in this model, the affinity of the two states differs and it is said that ligand binding stabilizes the state for which the ligand has higher affinity (Monod *et al.*, 1965). The conformational changes that this model describes are generally thought to reflect interactions among all subunits (i.e. quaternary structure) rather than the folding of each subunit (i.e. tertiary structure) In this model, activation is mediated by a transition from the inactive, resting state and the open channel conformation. Subsequent to these events, allosteric proteins undergo slower refractory or desensitization (on the order of tenths of seconds to minutes) processes. Further, this model accounts for a state of intermediate affinity (I), which occurs subsequent to receptor activation (A) but prior to the final desensitized state (D). This scheme suggests that the affinity of the receptor is low in the basal state (B), but higher in each of the subsequent transitory states, such that the rank order of affinity is D>I>A (Fig. 1-8A) (*see* Edelstein and Changeux, 1998). The extension of this model to account for desensitization processes without significant receptor activation, suggests that desensitization can be mediated by low concentrations of agonist that do not elicit significant current (Edelstein *et al.*, 1996).

A model that has been proposed to account for multiple classes of binding sites has been put forward by Raftery and colleagues (1983). In this scheme, binding to high and low affinity sites occurs by simple mechanisms, whereas channel activation requires cooperative interactions among different binding sites. The details of this model are adequate to explain activation and desensitization as parallel processes that are mediated by distinct binding sites on the receptor (Fig. 1-8B).

AIMS OF THE PRESENT STUDIES

It is the goal of the studies described in this thesis to characterize agonist-binding domains of GABA$_A$Rs using a multi-disciplinary approach that includes site-directed mutagenesis, radioligand binding, transient transfection and electrophysiology. These methods permit the substitution of amino acid residues in specific domains of the receptor macromolecule and allow for the evaluation of the consequences of those mutations, both in terms of binding and functional properties of a single receptor subtype. This technology better enables the researcher to investigate receptor domains, some of which had been previously investigated in brain homogenates using biochemical techniques such as photoaffinity labeling, chemical modification, and radioligand binding.

A number of investigators have provided detailed information on the nature of the low affinity GABA binding sites that mediate ion-channel activation, but there have been no reports of the structural basis of high affinity GABA/muscimol binding. However, the existence of these sites has been noted in a number of receptor preparations, including brain homogenates (*see* Olsen *et al.*, 1981; Agey and Dunn, 1989) and a stably transfected cell line (Davies *et al.*, 1994). A series of homology considerations based on the low affinity agonist-binding site the high affinity benzodiazepine-binding site, as described in detail in Chapter 2, suggested that a tyrosine residue in a particular domain ("loop" D) of the β2 subunit could contribute to the formation of this binding site. The primary hypothesis of this research is therefore that tyrosine-62 of the β2 subunit of the GABA$_A$R is an important determinant of high affinity agonist binding. The secondary hypothesis of Chapter 2 is that high affinity agonist binding does not play a major role in $GABA_AR$ ion-channel activation as has been suggested by others (Im *et al.*, 1997). However, an early model of nAChR activation and desensitization in which high affinity agonist-binding domains are reported to mediate desensitization of the receptor (Raftery *et al.*, 1983) is tested in Chapter 3 using mutants that alter high affinity agonist-binding properties. The aim of the studies described within Chapter 3 is therefore to determine the role of high affinity agonist binding in $GABA_AR$ desensitization processes. The elucidation of determinants of this binding domain has important consequences for those who study $GABA_AR$ function since the details of this work challenge the current dogma in the field that the high affinity binding sites simply represent the desensitized state of the sites required for ion-channel activation (Sigel and Buhr, 1997).

In Chapter 4, the goal of the study is to demonstrate that the partial inverse agonist, Ro15-4513, recognizes a domain of the receptor that is distinct from the positive allosteric modulator, flunitrazepam. Previous work from this laboratory has demonstrated that the primary site of photoincorporation of [^{3}H]flunitrazepam is H102 of the bovine α subunit (Duncalfe *et al.*, 1996). It was subsequently demonstrated the Ro15-4513 recognized a unique domain that was C-terminal to residue 125 of the $\alpha1$ subunit (Davies et al., 1996), an observation that was corroborated by a mutagenesis study of H101 (rat numbering) in which multiple amino acid substitutions differentially altered the affinity for flunitrazepam, but did not lead to a reduction in affinity for Ro15-4513. Further refinement of the localization of the Ro15-4513 labeling suggested that this compound labeled the domain between residues 247-281 of the $\alpha1$ subunit (Davies and Dunn, 1998). The best candidate for interaction with Ro15-4513 was the extracellular loop between TMII and TMIII, and this was the target for mutational analysis as described in this chapter. Therefore, the specific aim of the experiments described in Chapter 4 is to demonstrate that determinants within this region are important for Ro15-4513 binding and its effects. This may have important consequences for the pharmacotherapy of alcohol addiction since Ro15-4513 is reported to act as an alcohol antagonist and this region of the receptor may be important for the interactions of alcohol (Mihic *et al.*, 1997).

In Chapter 5, the interactions of zopiclone, a cyclopyrrolone that shares the same pharmacodynamic profile as the classical benzodiazepines, in investigated. The interactions of this compound with the GABA$_A$R are unclear as it does not appear to modulate the binding of other [^{3}H]ligands in the same manner as diazepam and kinetic data have suggested that the interaction of zopiclone with the GABA$_A$R occurs via a modulatory site distinct from classical benzodiazepines. There is also no clear consensus as to its efficacy at GABA$_A$Rs (see Chapter 5 for details). Therefore an investigation of its binding and functional properties was warranted. The goal of the work described in Chapter 5 was therefore to clarify the interaction of zopiclone with GABA$_A$R.

In conclusion, the global goal of the present study is to ascertain information as to amino acid residues that play a role in the binding of the agonists, muscimol and GABA, and benzodiazepine site ligands. The specific aims of these projects are (1) to identify determinants of high affinity agonist binding, (2) to elucidate the role of high affinity agonist binding in GABA$_A$R function, (3) to define further the novel domain between residues 247-283 of the $\alpha 1$ subunit that is photolabeled by Ro15-4513 and (4) to better define the interaction of zopiclone, a sedative-hypnotic, with GABA$_A$R.

Figure 1-1

(A) Schematic of a generic $GABA_AR$ subunit showing the large hydrophilic amino-terminus, the conserved cysteine-cysteine bridge (C139-C153), the four transmembrane domains (TMI-TMIV) and the short, extracellular carboxy terminal tail. Also shown are the relative positions of the six putative "loops" (labelled A-F) that are believed to form ligand recognition domains. (B) Linear model of a generic subunit showing the relative positions of the "loops" in the primary sequence of the protein, sites for *N*-linked glycosylation, the conserved cysteine loop, and the four transmembrane domains.

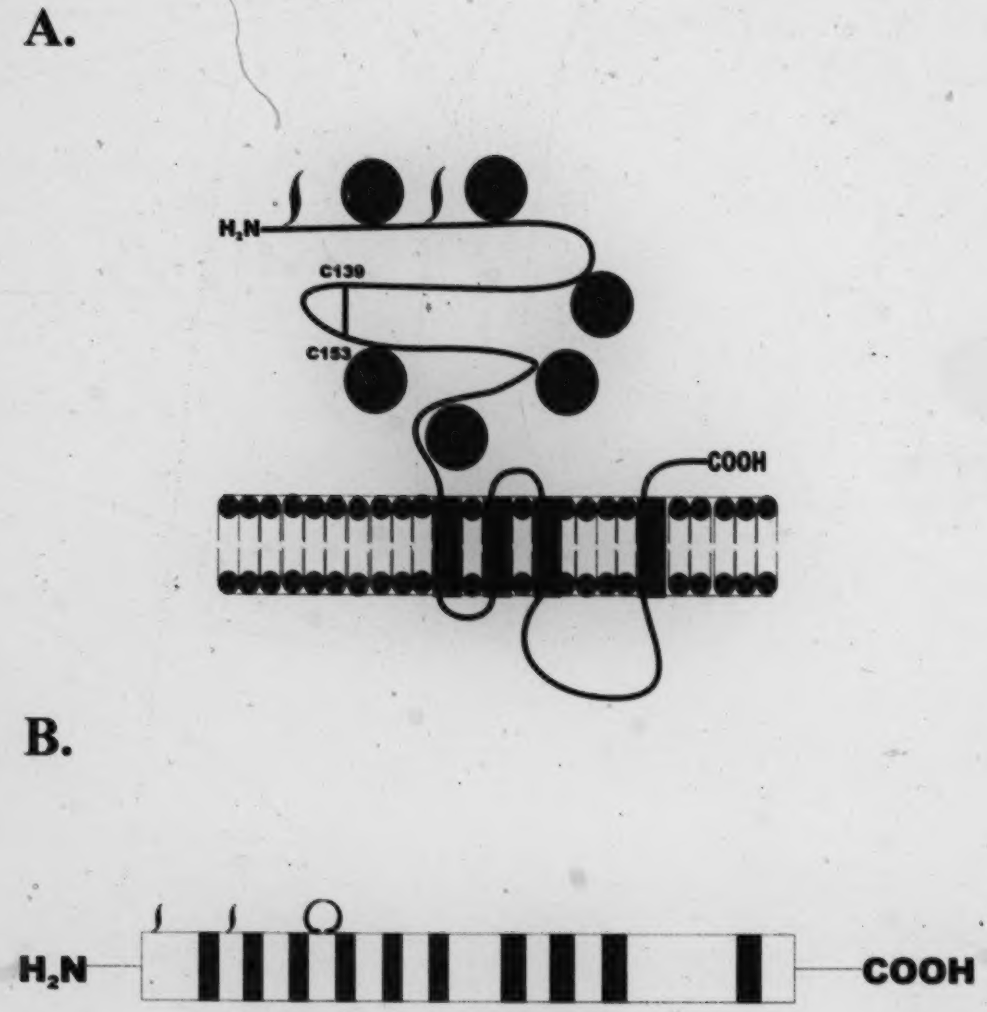

A.
H₂N
C139
C153
COOH
B.
H₂N
COOH

Figure 1-2

Model of the binding site of the nAChR, illustrating the importance of subunit-interfaces to the formation of recognition sites. Shown are the relative positions of amino acid residues from "loops" A-C of the α subunit and "loops" D-F on the adjacent γ or δ subunit. Also shown are the amino acids implicated in the formation of the acetylcholine/high affinity *d*-tubocurarine/low affinity α-conotoxin M-1 recognition site at the α-γ interface. These include αW86, αY93, αW149, αY151, αY190, αC192, αC193, αY198, αD200, γK34, γW55, γE57, γY111, γC115, γI116, γY117, γS161, γF172, γD174, and γE183. The numbering used is for the mature α and γ subunits of the nAChR, respectively. Homologous residues of the δ subunit have also been identified but have been omitted for clarity. Details of residues of the δ subunit that have been implicated at the α-δ interface are presented in the text

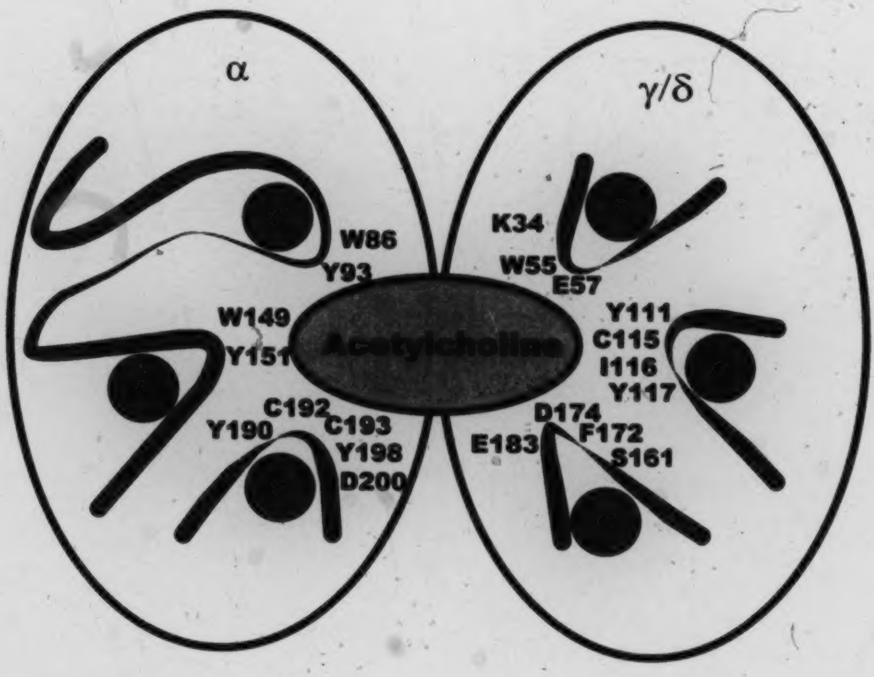

α
γ/δ
W86
Y93
W149
Y151
C192
Y190
C193
Y198
D200
Acetylcholine
K34
W55
E57
Y111
C115
I116
Y117
D174
F172
E183
S161

Figure 1-3

Partial amino acid sequence alignment for the α1-, β2-, and γ2-subunits of the rat $GABA_AR$, α, γ and δ subunits of the *Torpedo* nAChR subunits, α and β subunits of the rat GlyR, and the A subunit of the rat $5HT_3R$ from (A) "loops" A, (B) "loop" B, and (C) "loop" C. The numbering shown for "loop" A is for the mature α1 subunit of the $GABA_AR$, while the numbering for "loops" B and C corresponds to the mature β2 subunit of the $GABA_AR$. The amino acid residues in red lettering have been implicated in GABA benzodiazepine, dTC, DDF, acetylcholine, nicotine, serotonin and granisetron binding as detailed in the text. Residues that appear in the box represent conserved structural features of the LGIC subunits as indicated. For the purposes of alignment, the asterisks represent regions where the subunits are not of equivalent length and therefore lack a homologous residue at these positions.

A. Loop A

		92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
GABA$_A$R	α1	K	I	W	T	P	D	T	F	F	H	N	G	K	K	S	V	A
	β2	Q	L	W	V	P	D	T	Y	F	L	N	D	K	K	S	F	V
	γ2	K	I	W	I	P	D	T	F	F	R	N	S	K	K	A	D	A
nAChR	α	D	V	W	L	P	D	L	V	L	Y	N	N	A	D	G	D	F
	γ	L	L	W	L	P	D	V	V	L	E	N	N	V	D	G	Q	F
	δ	L	V	W	I	P	D	I	V	L	Q	N	N	N	D	G	Q	Y
GlyR	α1	S	I	W	K	P	D	L	F	F	A	N	E	K	G	A	H	F
	β	C	L	W	K	P	D	L	F	F	A	N	E	K	S	A	N	F
5HT$_3$R	A	S	I	W	V	P	D	I	L	I	N	E	F	V	D	V	G	•

B. Loop B

		153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169
GABA$_A$R	α1	K	F	G	S	Y	A	Y	T	R	A	E	V	V	Y	E	W	T
	β2	E	I	E	S	Y	G	Y	T	T	D	D	I	E	F	Y	W	R
	γ2	E	F	S	S	Y	G	Y	P	K	N	E	I	E	Y	K	W	K
nAChR	α	K	L	G	I	W	T	Y	D	G	T	K	V	S	I	S	P	E
	γ	V	F	R	S	Q	T	Y	N	A	H	E	V	N	L	Q	L	S
	δ	K	F	T	A	L	N	Y	D	A	N	E	I	T	M	D	L	M
GlyR	α1	Q	L	E	S	F	G	Y	T	M	N	D	L	I	F	E	W	Q
	β	Q	L	E	S	F	G	Y	T	T	D	D	L	R	F	I	W	Q
5HT$_3$R	A	T	F	T	S	W	L	H	T	I	Q	D	I	N	I	S	L	W

C. Loop C

		192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208
GABA$_A$R	α1	•	V	D	S	G	I	V	Q	S	S	T	G	E	Y	V	V	M
	β2	L	I	T	K	K	V	V	F	•	S	T	G	S	Y	P	R	L
	γ2	•	N	T	T	E	V	V	K	T	T	S	G	D	Y	V	V	M
nAChR	α	W	V	Y	Y	T	C	C	P	D	T	P	Y	L	•	•	D	I
	γ	K	N	Y	W	Q	L	T	D	K	D	D	T	D	F	•	Q	E
	δ	I	Y	•	P	D	K	F	P	N	G	T	N	Y	•	Q	D	V
GlyR	α1	L	R	Y	C	T	K	H	Y	N	•	T	G	K	F	T	C	I
	β	Y	G	N	C	T	K	Y	Y	K	G	T	G	Y	Y	T	C	V
5HT$_3$R	A	E	F	•	•	•	•	S	I	E	T	S	N	S	Y	A	E	M

Figure 1-4

Partial amino acid sequence alignment for the α1-, β2-, and γ2-subunits of the rat $GABA_AR$, α, γ and δ subunits of the *Torpedo* nAChR subunits, α and β subunits of the rat GlyR, and the A subunit of the rat $5HT_3R$ from (A) "loops" D, (B) "loop" E, and (C) "loop" F. The numbering shown for "loops" D and is for the mature β2 subunit, while the numbering for "loops" E corresponds to the mature α1 subunit of the $GABA_AR$. The numbering of "loop" F corresponds to the mature δ subunit of the nAChR. The amino acid residues in red lettering have been implicated in GABA benzodiazepine, *d*-tubocurarine, DDF, acetylcholine, nicotine, serotonin and granisetron binding as detailed in the text. Residues that appear in the box represent conserved structural features of the LGIC subunits as indicated.

A. Loop D

		58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74
GABA$_A$R	α1	T	I	D	V	F	F	R	Q	S	W	K	D	E	R	L	K	F
	β2	T	L	T	M	Y	F	Q	Q	A	W	R	D	K	R	L	S	Y
	γ2	T	I	D	I	F	F	A	Q	T	W	Y	D	R	R	L	K	F
nAChR	α	E	T	N	V	R	L	R	Q	Q	W	I	D	V	R	L	R	W
	γ	T	T	N	V	W	I	E	I	Q	W	N	D	Y	R	L	S	W
	δ	T	S	N	V	W	M	D	H	A	W	Y	D	H	R	L	T	W
GlyR	α1	R	V	N	I	F	L	R	Q	Q	W	N	D	P	R	L	A	Y
	β	R	V	N	I	F	L	R	Q	K	W	N	D	P	R	L	K	L
5HT$_3$R	A	T	T	Y	I	W	Y	R	Q	F	W	T	D	E	F	L	Q	W

B. Loop E

		114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130
GABA$_A$R	α1	M	P	N	K	L	L	R	I	T	D	G	T	L	L	Y	T	M
	β2	V	K	N	R	M	I	R	L	H	P	D	G	T	V	L	Y	G
	γ2	T	P	N	R	M	L	R	I	W	N	D	G	R	V	L	Y	T
nAChR	α	M	T	K	L	L	L	D	•	•	Y	T	G	K	I	M	W	T
	γ	Y	A	N	V	L	V	Y	N	D	G	S	M	Y	W	L	P	P
	δ	F	C	N	V	L	V	R	•	•	F	N	G	Y	V	T	W	L
GlyR	α1	T	D	N	K	L	L	R	I	S	R	N	G	N	V	L	Y	S
	β	Q	E	N	I	L	L	F	I	F	R	D	G	D	V	I	V	S
5HT$_3$R	A	P	Y	V	Y	V	H	H	Q	G	E	V	Q	N	Y	K	P	L

C. Loop F

		163	164	165	166	167	178	179	180	181	182	183	184	185	186	187	188	189
GABA$_A$R	α1	V	Y	E	W	T	V	V	V	A	E	D	G	S	•	R	L	N
	β2	I	E	F	Y	W	V	•	V	T	G	V	K	I	E	L	P	Q
	γ2	E	I	V	Y	Q	•	E	V	G	D	T	R	S	W	L	Y	Q
nAChR	α	S	I	S	P	E	R	•	D	L	S	T	F	M	E	S	G	E
	γ	N	L	Q	L	S	H	I	D	P	E	D	F	T	E	N	G	E
	δ	T	M	D	L	M	I	I	D	P	E	A	F	T	E	N	G	E
GlyR	α1	I	F	E	W	Q	V	V	Q	V	A	D	G	L	T	L	P	Q
	β	R	F	I	W	Q	K	V	Q	L	•	E	K	I	A	L	P	Q
5HT$_3$R	A	N	I	S	L	W	R	•	D	K	S	I	F	I	N	Q	G	E

Figure 1-5

Model of the benzodiazepine binding site at the interface of adjacent α–γ subunits of the $GABA_AR$. The model illustrates the relative positions of the amino acid residues that have been implicated in agonist, antagonist, and inverse agonist recognition. Also shown are the amino acids implicated in the formation of the high affinity benzodiazepine site. These include αH101, αY159, αT162, αG200, αS204, αT206, αY209, αV211, γM57, γY58, γF77, γYA79, and γM130. The numbering used is for the mature α1 and γ2 subunits, respectively. "Loop" F is not shaded in red because no residues from this loop have been implicated in the formation of the benzodiazepine site to date.

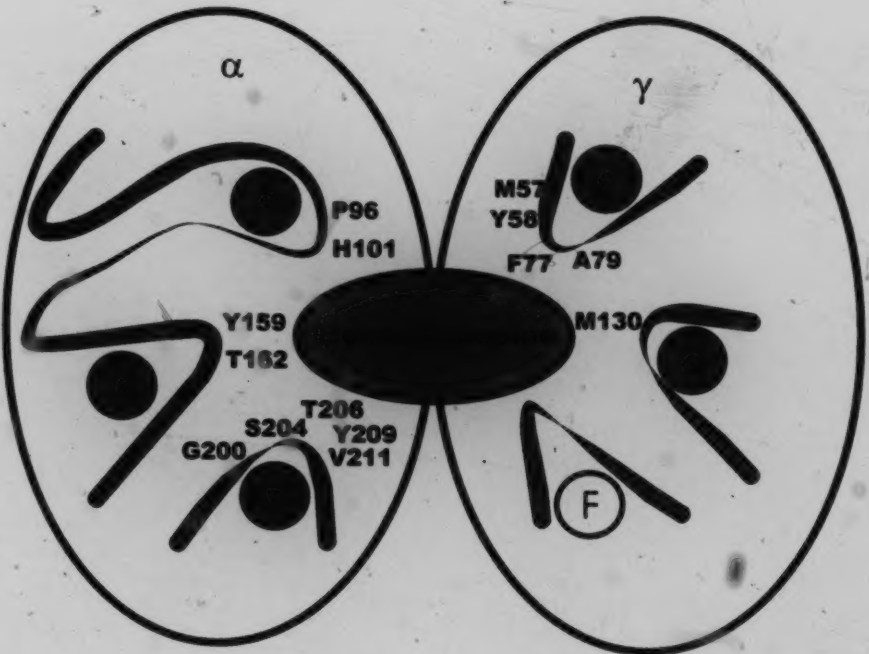

α
γ
P96
H101
Y159
T162
T206
S204
Y209
G200
V211
M57
Y58
F77
A79
M130
F

Figure 1-6

Model of the low affinity binding site at the interface of adjacent β–α subunits of the $GABA_AR$. The model illustrates the relative positions of the amino acid residues that have been implicated in agonist and antagonist recognition. Also shown are the amino acids implicated in the formation of the low affinity GABA binding (i.e channel activation) site. These include βY157, βT161, βT202, βY205, αF64, αR66, αS68, αR120, and αI121. The numbering used is for the mature β2 and α1 subunits, respectively. "Loops" A and F are not shaded in red because no residues from these loops have been implicated in the formation of the GABA site to date.

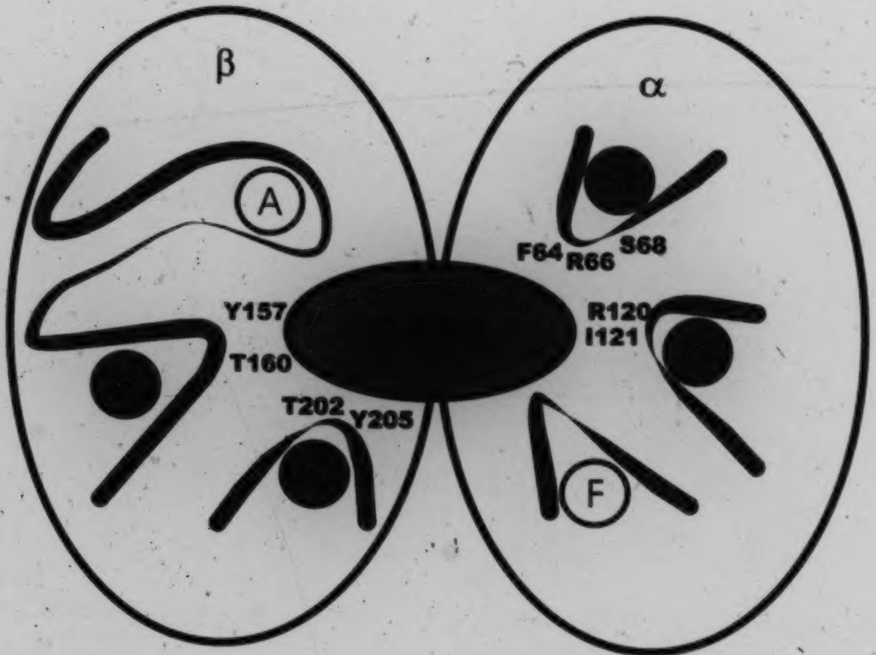
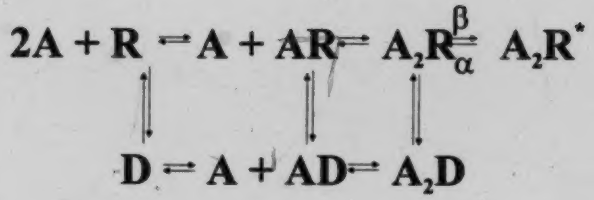

β
α
A
F64
R66
S68
Y157
T160
R120
I121
T202
Y205
F

Figure 1-7

Models of GABA$_A$R activation and desensitization as proposed by (A) Del Castillo and Katz (1957) and (B) Jones *et al.* (1998). In (A), there are two agonist (A) binding sites per receptor (R), both of which must be occupied before isomerization to the active state (*). The desensitized (D) state of the receptor can be achieved following ion channel opening or from the mono-liganded state. In (B), the open channel can be achieved via the mono- or di-liganded states, which give rise to different states of desensitization and activation. Detailed descriptions of each model are presented in the text.

A.


2A + R
A + AR
A2R
A2R*
β
α
D
A + AD
A2D

B.

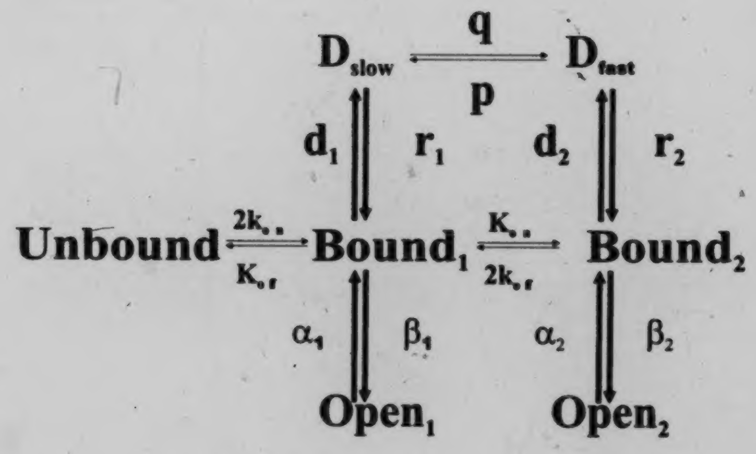
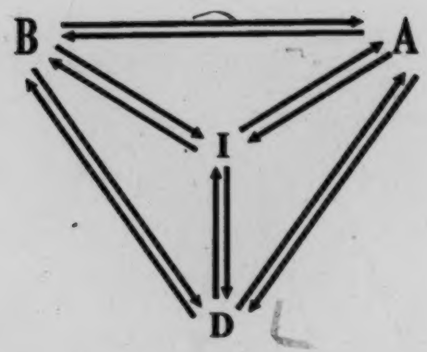

Dslow
Dfast
q
p
d1
r1
d2
r2
Unbound
Bound1
Bound2
2kon
Koff
Kon
2koff
α1
β1
α2
β2
Open1
Open2

Figure 1-8

Models of $GABA_AR$ activation and desensitization as proposed by (A) Heidmann and Changeux (1980) and (B) Raftery *et al.* (1983). In (A), B represents the basal or resting state and A represents the active state. The intermediate (I) and desensitized (D) states are achieved through the open channel state. In (B), ligand binding to two high (H) affinity sites in (1) allows the receptor to reach the closed, desensitized state, whereas the open channel state is reached by the sequential binding of two agonist molecules as depicted in (2) to the low affinity sites. The desensitized state can also be achieved through this pathway. Detailed descriptions of each model are presented in the text.

A.



B.

$$(1)\ 2R_H + 2A \overset{K = 10nM}{\rightleftharpoons} 2R_{HA} \rightleftharpoons 2R_{HA*\,(\text{desensitized})}$$

$$(2)\ 2R_L + 2A \overset{K = 100\,\mu M}{\rightleftharpoons} 2R_{LA} \rightleftharpoons 2R_{LA}{}^{*}{}_{(\text{open})}$$

Table 1-1. Relative equilibrium dissociation constants (K_D) and binding densities (B_{max}) for [^{3}H]GABA or [^{3}H]muscimol binding at high (H) and low (L) affinity sites. In cases where K_D and B_{max} values are reported from multiple brain regions, only values for binding from bovine cortex are presented. ([*]Data represent the mean ± SEM; [#]Data represent the mean ± SD; N.D., not determined).

Reference	Ligand	$K_{D(H)}$ (nM)	$Bmax_{(H)}$ (pmol/mg)	$K_{D(L)}$ (nM)	$Bmax_{(L)}$ (pmol/mg)
Guidotti *et al.*, 1978	GABA	28	N.D.	111	N.D.
Greenlee *et al.*, 1978[#]	GABA	24 ± 6	0.69	150 ± 9.7	2.90 ± 0.62
Enna and Snyder, 1975	GABA	N.D.	N.D.	1200[@]	30
Guidotti *et al.*, 1979	GABA	20	1.00	120	0.75
Olsen *et al.*, 1981	GABA	13 ± 6	0.33 ± 0.17	300 ± 150	1.8 ± 0.60
Kurioka *et al.*, 1981	GABA	18.6	2.8	60.5	4.4
Kurioka *et al.*, 1981	GABA	3500	72.1	18200	190.8
Madtes Jr., 1984	GABA	0.75	0.50	113.9	2.88
Madtes Jr. *et al.*, 1986[*]	GABA	18.0 ± 2.0	0.064±0.02	N.D.	N.D.
Costa *et al.*, 1990[*]	GABA	10.7 ± 0.9	1.7 ± 0.2	131 ± 20	4.5 ± 0.3
Shank *et al.*, 1990[&]	GABA	16.3	0.88	224	1.12
Fiszer de Plazas *et al.*, 1993[*]	GABA	7.1± 0.2	0.99 ± 0.02	113.4±8.6	6.90 ± 0.3
Wang *et al.*, 1979	muscimol	9	2.1	70	0.7
Williams and Risley, 1979	muscimol	4.5	0.51	30	0.99
Madtes Jr. *et al.*, 1986[*]	muscimol	19.1 ± 3.2	0.4 ±0.08	N.D.	N.D.
Madtes Jr. *et al.*, 1989[*]	muscimol	7.6 ± 1.02	0.3 ± 0.04	104 ± 43	2.25 ± 0.5
Yang and Olsen, 1987	muscimol	20	1.0	365	2.5
Agey and Dunn, 1989	muscimol	7	3.5	300	6.2
Newell *et al.*, 2000[*]	muscimol	8.9 ± 0.5	N.D.	429 ± 40	N.D.

[@] sodium dependent binding (K_D for sodium independent binding = 370 nM)

[&] Also reported is an ultralow affinity binding site (K_D = 34 μM, B_{max} = 90 pmol/mg)

BIBLIOGRAPHY

ABRAMSON, S.N., LI, Y., CULVER, P. AND TAYLOR, P. (1989). An analog of lophotoxin reacts covalently with Tyr^{190} in the α–subunit of the nicotinic acetylcholine receptor. *J. Biol. Chem.*, **264**, 12666-12672.

AGEY, M.W. AND DUNN, S.M.J. (1989). Kinetics of [^{3}H]muscimol binding to the GABA$_A$ receptor in bovine brain membranes. *Biochemistry*, **28**, 4200-4208.

AMIN, J. AND WEISS, D.S. (1993). GABA$_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature*, **366**, 565-569.

AMIN, J., DICKERSON, I.M. AND WEISS, D.S. (1994). The agonist binding site of the γ-aminobutyric acid type A channel is not formed by the extracellular cysteine loop. *Mol. Pharmacol.*, **45**, 317-323.

AMIN, J., BROOKS-KAYAL, A. AND WEISS, D.S. (1997). Two tyrosine residues on the α subunit are crucial for benzodiazepine binding and allosteric modulation of γ-aminobutyric acid$_A$ receptors. *Mol. Pharmacol.*, **51**, 833-841.

ANGELOTTI, T.,P. AND MACDONALD, R.L. (1993). Assembly of GABA$_A$ receptor subunits: $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ subunits produce unique ion channels with dissimilar single-channel properties. *J. Neurosci.*, **13**, 1429-1440.

ANGELOTTI, T.P., UHLER, M.D. AND MACDONALD, R.L. (1993). Assembly of GABA$_A$ receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J. Neurosci.*, **13**, 1418-1428.

ARAUJO, F., TAN, S., RUANO, D., BENAVIDES, J., VITORICA, J. AND SCHOEMAKER, H. (1996). Molecular and pharmacological characterization of native cortical γ-aminobutyric acid$_A$ receptors containing both α_1 and α_3 subunits. *J. Biol. Chem.*, **271**, 27902-27911.

ARBILLA, S., DEPOORTERE, H., GEORGE, P., AND LANGER S.Z. (1985). Pharmacological profile of the imidazopyridine zolpidem at benzodiazepine receptors and electrocorticogram in rats. *Naun. Schmied. Arch. Pharmacol.*, **330**, 248-251.

AYLWIN, M.L. AND WHITE, M.M. (1994). Ligand-receptor interactions in the nicotinic acetylcholine receptor probed using multiple substitutions at conserved tyrosines on the α subunit. *FEBS Lett.*, **349**, 99-103.

BACKUS, K.H., ARIGONI, M., DRESCHER, U., SCHEURER, L., MALHERBE, P., MÖHLER, H. AND BENSON, J.A. (1993). Stoichiometry of a recombinant GABA$_A$ receptor deduced from mutation-induced rectification. *NeuroReport*, **5**, 285-288.

BARNES, E.M. JR. (2000). Intracellular trafficking of GABA$_A$ receptors. *Life Sci.*, **66**, 1063-1070.

BATESON, A.N., LASHAM, A. AND DARLISON, M.G. (1991). γ-Aminobutyric acid$_A$ receptor heterogeneity is increased by alternative splicing of a novel β-subunit gene transcript. *J. Neurochem.*, **56**, 1437-1440.

BEAUMONT, K., CHILTON, W.S., YAMAMURA, H.I. AND ENNA, S.J. (1978). Muscimol binding in rat brain: association with synaptic GABA receptors. *Brain Res.*, **148**, 153-162.

BELELLI, D., PAU, D., CABRAS, G., PETERS, J.A. AND LAMBERT, J.J. (1997). A single amino acid confers barbiturate sensitivity upon the GABA ρ1 receptor. *Br. J. Pharmacol.*, **127**, 601-604.

BELELLI, D, PISTIS, M., PETERS, J.A. AND LAMBERT, J.J. (1999). The interaction of general anesthetics and neurosteroids with $GABA_A$ and glycine receptors. *Neurochem. Int.*, **34**, 447-452.

BENKE, D., FRITSCHY, J.-M., TRZECIAK, A., BANNWARTH, W. AND MOHLER, H. (1994). Distribution, prevalence, and drug binding profile of γ-aminobutyric acid type A receptor subtypes differing in the β–subunit variant. *J. Biol. Chem.*, **269**, 27100-27107.

BENKE, D., HONER, M., MICHEL, C. AND MÖHLER, H. (1996). $GABA_A$ receptor subtypes differentiated by their γ-subunit variants: prevalence, pharmacology, and subunit architecture. *Neuropharmacology*, **35**, 1413-1423.

BIRNIR, B., TIERNEY, M.I., PILLAI, N.P., COX, G.B. AND GAGE, P.W. (1995). Rapid desensitization of α1β1 $GABA_A$ receptors expressed in Sf9 cells under optimized conditions. *J. Membr. Biol*, **148**, 193-202.

BLAIR, L.E.A., LEVITAN, E.S., MARSHALL, J., DIONNE, V.E. AND BARNARD, E.A. (1988). Single subunits of the $GABA_A$ receptor form ion channels with properties of the native receptor. *Science*, **242**, 577-579.

BLOUNT, P. AND MERLIE, J.P. (1989). Molecular basis of the two nonequivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. *Neuron*, **3**, 349-357.

BLOUNT, P., SMITH, M.M. AND MERLIE, J.P. (1990). Assembly intermediates of the mouse muscle nicotinic acetylcholine receptor in stably transfected fibroblasts. *J. Cell Biol.*, **111**, 2601-2611.

BOESS, F.G., STEWARD, L.J., STEELE, J.A., LIU, D., REID, J., GLENCORSE, T.A. AND MARTIN, I.L. (1997). Analysis of the ligand binding site of the $5\text{-}HT_3$ receptor using site-directed mutagenesis: importance of glutamate 106. *Neuropharmacology*, **36**, 637-647.

BOILEAU, A.J., KUCKEN, A.M., EVERS, A.R. AND CZAJKOWSKI, C. (1998). Molecular dissection of benzodiazepine binding and allosteric coupling using chimeric γ-aminobutyric acid_A receptor subunits. *Mol. Pharmacol.*, **53**, 295-303.

BOILEAU, A.J. AND CZAJKOWSKI, C. (1999). Identification of transduction elements for benzodiazepine modulation of the $GABA_A$ receptor: three residues are required for allosteric coupling. *J. Neurosci.*, **19**,10213-10220.

BOILEAU, A.J., EVERS, A.R., DAVIS, A.F. AND CZAJKOWSKI, C. (1999). Mapping the agonist binding site of the $GABA_A$ receptor: evidence for a β-strand. *J. Neurosci.*, **19**, 4847-4854.

BREN, N. AND SINE, S.M. (1997). Identification of residues in the adult nicotinic acetylcholine receptor that confer selectivity for curariform antagonists. *J. Biol. Chem*, **272**, 30793-30798.

BRISTOW, D.R., MORATALLA, R. AND MARTIN, I.L. (1990). Flunitrazepam increases the affinity of the $GABA_A$ receptor in cryostat-cut rat brain sections. *Eur. J. Pharmacol.*, **184**, 339-340.

BROWNING, M.D., BUREAU, M., DUDEK, E.M. AND OLSEN, R.W. (1990). Protein kinase C and cAMP-dependent protein kinase phosphorylate the β subunit of the purified γ-aminobutyric acid A receptor. *Proc. Natl. Acad. Sci U.S.A.*, **87**, 1315-1318.

BUHR, A., BAUR, R., MALHERBE, P. AND SIGEL, E. (1996). Point mutations of the $\alpha 1\beta 2\gamma 2$ γ-aminobutyric acid_A receptor affecting the modulation of the channel by ligands of the benzodiazepine binding site. *Mol. Pharmacol.*, **49**, 1080-1084.

BUHR, A., SCHAERER, M.T., BAUR, R. AND SIGEL, E. (1997a). Residues at position 206 and 209 of the $\alpha 1$ subunit of γ-aminobutyric acid$_A$ receptors influence affinities for benzodiazepine binding site ligands. *Mol. Pharmacol.*, **52**, 676-686.

BUHR, A., BAUR, R. AND SIGEL, E. (1997b). Subtle changes in residue 77 of the γ subunit of the $\alpha 1\beta 2\gamma 2$ GABA$_A$ receptors drastically alter the affinity for ligands of the benzodiazepine binding site. *J. Biol. Chem.*, **272**, 11799-11804.

BULLER, A.L., HASTINGS, G.A., KIRKNESS, E.F. AND FRASER, C.M. (1994). Site-directed mutagenesis of *N*-linked glycosylation sites of the γ-aminobutyric acid type A receptor subunit. *Mol. Pharmacol.*, **46**, 858-865.

BURCH, T.R., THYGARAJAN, R. AND TICKU, M.K. (1982). Group-selective reagent modification of the benzodiazepine-γ-aminobutyric acid receptor-ionophore complex reveals that low affinity γ-aminobutyric acid receptors stimulate benzodiazepine binding. *Mol. Pharmacol.*, **23**, 52-59.

BUREAU, M. AND OLSEN, R.W. (1988). γ-Aminobutyric acid/benzodiazepine receptor protein carries binding sites for both ligands on both two major peptide subunits. *Biochem. Biophys. Res. Comm.*, **153**, 1006-1011.

BUREAU, M. AND OLSEN, R.W. (1990). Multiple subunits of the γ-aminobutyric acid-A receptor protein show different ligand-binding affinities. *Mol. Pharmacol.*, **37**, 497-502.

BUREAU, M. AND OLSEN, R.W. (1993). GABA$_A$ receptor subtypes: ligand binding heterogeneity demonstrated by photolabeling and autoradiography. *J. Neurochem.*, **61**, 1479-1491.

CASALOTTI, S.O., STEPHENSON, F.A. AND BARNARD, E.A. (1986). Separate subunits for agonist and benzodiazepine binding in the γ-aminobutyric $acid_A$ receptor oligomers. *J. Biol. Chem.*, **261**, 15013-15016.

CASH, D.J. AND SUBBARAO, K. (1987a). Desensitization of γ–aminobutyric acid receptor from rat brain: two distinguishable receptors on the same membrane. *Biochemistry*, **26**, 7556-7562.

CASH, D.J. AND SUBBARAO, K. (1987b). Two desensitization processes of GABA receptor from rat brain: rapid measurements of chloride ion flux using quench flow techniques. *FEBS Lett.*, **217**, 129-133.

CAVALLA, D. AND NEFF, N.H. (1985). Photoaffinity labeling of the $GABA_A$ receptor with [^{3}H]muscimol. *J. Neurochem.*, **44**, 916-921.

CHANG, Y., WANG, R., BAROT, S. AND WEISS, D.S. (1996). Stoichiometry of a recombinant $GABA_A$ receptor. *J. Neurosci.*, **16**, 5415-5424.

CHANGEUX, J.-P. AND EDELSTEIN, S.J. (1998). Allosteric receptors after 30 years. *Neuron*, **21**, 959-980.

CHERUBINI, E., GAIARSA, J.L. AND BEN-ARI. Y. (1991). GABA: an excitatory transmitter in early postnatal life. *Trends Neurosci*, **14**, 515-519.

CHIARA, D.C. AND COHEN, J.B. (1997). Identification of amino acids contributing to high and low affinity *d*-tubocurarine sites in the *Torpedo* nicotinic acetylcholine receptor. *J. Biol. Chem.*, **272**, 32940-32950.

CHIARA, D.C., MIDDLETON, R.E. AND COHEN, J.B. (1998). Identification of tryptophan 55 as the primary site of [^{3}H]nicotine photoincorporation in the γ-subunit of the *Torpedo* nicotinic acetylcholine receptor. *FEBS Lett.*, **423**, 223-226.

CHIARA, D.C., XIE, Y. AND COHEN, J.B. (1999). Structure of the agonist-binding sites of the *Torpedo* nicotinic acetylcholine receptor: affinity-labeling and mutational analyses identify γTyr-111/δArg-113 as antagonist affinity determinants. *Biochemistry*, **38**, 6689-6698.

CLEMENTS, J.D. (1996). Transmitter timecourse in the synaptic cleft: its role in central synaptic function. *Trends Neurosci.*, **19**, 163-171.

COCKROFT, V.B., OSGUTHORPE, D.J., BARNARD, E.A. AND LUNT, G.G. (1990). Modeling of agonist binding to the ligand-gated ion channel superfamily of receptors. *Proteins: Struc. Func. Genet.*, **8**, 386-397.

COHEN, J.B., SHARP, S.D. AND LIU, W.S. (1991). Structure of agonist-binding site of the nicotinic acetylcholine receptor: [^{3}H]acetylcholine mustard identifies residues in the cation-binding subunit. *J. Biol. Chem.*, **266**, 23354-23364.

COLQUHOUN, D. (1998). Binding, gating, affinity, and efficacy: the interpretation of structure-activity relationships for agonists and the effects of mutating receptors. *Br. J. Pharmacol.*, **125**, 924-947.

CONCAS, A., SERRA, M., CRISPONI, G., NURCHI, V., CORDA, M.G. AND BIGGIO, G. (1984). Changes in the characteristics of low affinity GABA binding sites elicited by Ro15-1788. *Life Sci.*, **36**, 329-337.

CONNOLLY, C.N., KRISHEK, B.J., MCDONALD, B.J., SMART, T.G. AND MOSS, S.J. (1996). Assembly and cell surface expression of heteromeric and homomeric γ-aminobutyric acid type A receptors. *J. Biol. Chem.*, **271**, 89-96.

CONNOLLY, C.N., UREN, J.M., THOMAS, P., GORRIE, G.H., GIBSON, A., SMART, T.G. AND MOSS, S.J. (1999). Subcellular localization and endocytosis of homomeric γ2 subunit splice variants of γ-aminobutyric acid type A receptors. *Mol. Cell. Neurosci.*, **13**, 259-271.

CONNOR, J.X., BOILEAU, A.J. AND CZAJKOWSKI, C. (1998). A $GABA_A$ receptor α_1 subunit tagged with green fluorescent protein requires a β subunit for functional cell surface expression. *J. Biol. Chem.*, **273**, 28906-28911.

CORDA, M.G., SANNA, E., CONCAS, A., GIORGI, O., ONGINI, E., NURCHI, V., PINTORI, T., CRISPONI, G. AND BIGGIO, G. (1986). Enhancement of γ-aminobutyric acid binding by quazepam, a benzodiazepine derivative with preferential affinity for type I benzodiazepine receptors. *J. Neurochem.*, **47**, 370-374.

CORDA, M.G. GIORGI, O., LONGONI, B., MEREU, G.P. AND BIGGIO, G. (1987). Enhancement of γ-aminobutyric acid binding by the anxiolytic β-carbolines ZK 93423 and ZK 91296. *J. Neurochem.*, **48**, 1355-1358.

COSTA, E., VESCI, L., FONTANA, M., SOLINAS, S.P., DUPRE, S. AND CAVALLINI, D. (1990). Displacement of $[^3H]$GABA binding to bovine brain receptor by sulfur containing analogues. *Neurochem. Int.*, **17**, 547-551.

CZAJKOWSKI, C. AND KARLIN, A. (1991). Agonist binding site of *Torpedo* electric tissue nicotinic acetylcholine receptor: a negatively charged region of the δ subunit within 0.9 nm of the α subunit binding disulfide. *J. Biol. Chem.*, **266**, 22603-22612.

CZAJKOWSKI, C. AND KARLIN, A. (1993). Negatively charged amino acid residues in the nicotinic acetylcholine receptor δ subunit that contribute to the binding of acetylcholine. *Proc. Natl. Acad. Sci U.S.A.*, **90**, 6285-6289.

CZAJKOWSKI, C. AND KARLIN, A. (1995). Structure of the nicotinic acetylcholine-binding site: identification of acidic residues in the δ subunit within 0.9 nm of the α-subunit binding disulfide. *J. Biol. Chem.*, **270**, 3160-3164.

DALZIEL, J.E., COX, G.B., GAGE, P.W. AND BIRNIR, B. (1999). Mutant human $\alpha_1\beta_1$(T262Q) $GABA_A$ receptors are directly activated but not modulated by pentobarbital. *Eur. J. Pharmacol.*, **385**, 283-286.

DAVIES, M., STEELE, J.A., HADINGHAM, K.L., WHITING, P.J. AND DUNN, S.M.J. (1994). Characterization of a permanently transfected cell line expressing the human α1β1γ2S $GABA_A$ receptor subtype. *Can. J. Physiol. Pharmacol.*, **72**, 337.

DAVIES, M., BATESON, A.N. AND DUNN, S.M.J. (1996). Molecular biology of the $GABA_A$ receptor: functional domains implicated by mutational analysis. *Frontiers Biosci.*, **1**, d214-233.

DAVIES, M., MARTIN, I.L., BATESON, A.N., HADINGHAM, K.L., WHITING, P.J. AND DUNN, S.M.J. (1996). Identification of domains in human recombinant $GABA_A$ receptors that are photoaffinity labelled by [^{3}H]flunitrazepam and [^{3}H]Ro15-4513. *Neuropharmacology*, **35**, 1199-1208.

DAVIES, M. AND DUNN, S.M.J. (1998). Identification of a unique domain in bovine brain $GABA_A$ receptor that is photoaffinity labelled by [^{3}H]Ro15-4513. *Biochem. Biophys. Res. Comm.*, **246**, 650-653.

DAVIES, M., BATESON, A.N. AND DUNN, S.M.J. (1998a). Structural requirements for ligand interactions at the benzodiazepine recognition site of the $GABA_A$ receptor. *J. Neurochem.*, **70**, 2188-2194.

DAVIES, M., THUYNSMA, R.P. AND DUNN, S.M.J. (1998b). Effects of propofol and pentobarbital on ligand binding to $GABA_A$ receptors suggest a similar mechanism of action. *Can. J. Physiol. Pharmacol.*, **76**, 46-52.

DAVIES, M., NEWELL, J.G., DERRY, J.M.C., MARTIN, I.L. AND DUNN, S.M.J. (2000). Characterization of the interaction of zopiclone with γ-aminobutyric acid type A receptors. *Mol. Pharmacol.*, **54**, in press.

DAVIES, P.A., PISTIS, M., HANNA, M.C., PETERS, J.A., LAMBERT, J.J., HALES, T.G. AND KIRKNESS, E.F. (1999). The $5HT_{3B}$ subunit is a major determinant of serotonin-receptor function. *Nature*, **397**, 259-363.

DENG, L., RANSOM, R.W. AND OLSEN, R.W. (1986). [^{3}H]Muscimol photolabels the γ-aminobutyric acid receptor binding site on a peptide subunit distinct from that labeled with benzodiazepines. *Biochem. Biophys. Res. Comm.*, **138**, 1308-1314.

DENNIS, M., GIRAUDAT, J., KOTZYBA-HIBERT, F., GOELDNER, M., HIRTH, C., CHANG, J.-Y., LAZURE, C., CHRÉTIEN, M. AND CHANGEUX, J.-P. (1988). Amino acids of the *Torpedo marmorata* acetylcholine receptor α subunit labeled by a photoaffinity ligand for the acetylcholine binding site. *Biochemistry*, **27**, 2346-2357.

DOBLE, A. AND MARTIN, I.L. (1992). Multiple benzodiazepine receptors: no reason for anxiety. *Trends Pharmacol. Sci.*, **13**, 76-81.

DUGGAN, M.J, AND STEPHENSON, F.A. (1990). Biochemical evidence for the existence of γ-aminobutyrate$_A$ receptor iso-oligomers. *J. Biol. Chem.*, **265**, 3831-3835.

DUGGAN, M.J., POLLARD, S. AND STEPHENSON, F.A. (1991). Immunoaffinity purification of $GABA_A$ receptor α-subunit iso-oligomers: demonstration of receptor populations containing α1α2, α1α3, and α2α3 subunit pairs. *J. Biol. Chem.*, **266**, 24778-24784.

DUNCALFE, L.L., CARPENTER, M.R., SMILLIE, L.B., MARTIN, I.L. AND DUNN, S.M.J. (1996). The major site of photoaffinity labeling of the $GABA_A$ receptor by $[^3H]$flunitrazepam is histidine 102 of the α subunit. *J. Biol. Chem.*, **271**, 9209-9214.

DUNN, S.M.J. AND RAFTERY, M.A. (1982). Activation and desensitization of *Torpedo* acetylcholine receptor: evidence for separate binding sites. *Proc. Natl. Acad. Sci., U.S.A.*, **79**, 6757-6761.

DUNN, S.M.J., DAVIES, M., MUNTONI, A.L. AND LAMBERT, J.J. (1999). Mutagenesis of the rat α1 subunit of the γ-aminobutyric $acid_A$ receptor reveals the importance of residue 101 in determining allosteric effects of benzodiazepine site ligands. *Mol. Pharmacol.*, **56**, 768-774.

EBERT, B., WAFFORD, K.A., WHITING, P.J., KROGRGAARD-LARSEN, P. AND KEMP, J.A. (1994). Molecular pharmacology of γ-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α, β, and γ receptor combinations. *Mol. Pharmacol.*, **46**, 957-963.

EBERT, B., THOMPSON, S.A., SAOUNATSHOU, K., MCKERNAN, R., KROGSGAARD-LARSEN, P. AND WAFFORD, K.A. (1997). Differences in agonist/antagonist binding affinity and receptor transduction using recombinant human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.*, **52**, 1150-1156.

EDELSTEIN, S.J., SCHAAD, O., HENRY, E., BERTRAND, D. AND CHANGEUX, J.-P. (1996). A kinetic mechanism for nicotinic acetylcholine receptors based on multiple allosteric transitions. *Biol. Cybern.*, **75**, 361-379.

ENNA, S.J. AND SNYDER, S.H. (1975). Properties of γ-aminobutyric acid (GABA) receptor binding in rat brain synaptic membrane fractions. *Brain Res.*, **100**, 81-97.

ENNA, S.J. AND SNYDER, S.H. (1977). Influences of ions, enzymes, and detergents on γ-aminobutyric acid-receptor binding in synaptic membranes of rat brain. *Mol. Pharmacol.*, **13**, 442-453.

FARRAR, S.J., WHITING, P.J., BONNERT, T.P. AND MCKERNAN, R.M. (1999). Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. *J. Biol. Chem.*, **274**, 10100-10104.

FILIPOVA, N., SEDELNIKOVA, A., ZONG, Y., FORTINBERRY, H. AND WEISS, D.S. (2000). Regulation of recombinant γ-aminobutyric acid $(GABA)_A$ and $GABA_C$ receptors by protein kinase C. *Mol. Pharmacol.*, **57**, 847-856.

FISHER, J.L., ZHANG, J. AND MACDONALD, R.L. (1997). The role of α1 and α6 subtype amino-terminal domains in allosteric regulation of γ-aminobutyric $acid_a$ receptors. *Mol. Pharmacol.*, **52**, 714-724.

FISHER, J.L. AND MACDONALD, R.L. (1998). The role of an α subtype M2-M3 his in regulating inhibition of $GABA_A$ receptor current by zinc and other divalent cations. *J. Neurosci.*, **18**, 2944-2953.

FISZER DE PLAZAS, S. AND MITDRIDATE DE NOVARA, A. (1990). Effect of diazepam on the low affinity GABA binding sites at different developmental stages of the chick optic lobe. *Neurochem. Int.*, **17**, 381-387.

FISZER DE PLAZAS, S., GRAVIELLE, M.C., MITRIDATE DE NOVARA, A. AND FLORES, V. (1993). Methods for removing endogenous factors from CNS membrane preparations: differences in [^{3}H]GABA binding parameters and developmental-related effects. *Neurochem. Res.*, **18**, 385-391.

FU, D.-X. AND SINE, S.M. (1994). Competitive antagonists bridge the α-γ subunit interface of the acetylcholine receptor through quaternary ammonium–aromatic interactions. *J. Biol. Chem.*, **269**, 26152-26157.

FUKAMI, S., UCHIDA, I., TAKENOSHITA, M., MASHIMO, T. AND YOSHIYA, I. (1999). The effects of a point mutation of the β2 subunit of $GABA_A$ receptor on direct and modulatory actions of general anaesthetics. *Eur. J. Pharmacol.*, **268**, 269-276.

GALLAGER, D.W., RAUCH, S.L. AND MALCOLM, A.B. (1984). Alterations in a low affinity GABA recognition site following chronic benzodiazepine treatment. *Eur. J. Pharmacol.*, **98**, 159-160.

GALZI, J.-L., REVAH, F., BLACK, D., GOELDNER, M., HIRTH, C. AND CHANGEUX, J.-P. (1990). Identification of a novel amino acid α-tyrosine 93 within the cholinergic ligands-binding sites of the acetylcholine receptor by photoaffinity labeling: additional evidence for a three-loop model of the cholinergic-ligands binding sites. *J. Biol. Chem.*, **265**, 10430-10437.

GELMAN, M.S., CHANG, W., THOMAS, D.Y., BERGERON, J.J.M. AND PRIVES, J.M. (1995). Role of the endoplasmic reticulum chaperone calnexin in subunit folding and assembly of the nicotinic acetylcholine receptors. *J. Biol. Chem.*, **270**, 15085-15092.

GORRIE, G.H., VALLIS, Y., STEPHENSON, A., WHITFIELD, J., BROWNING, B., SMART, T.G. AND MOSS, S.J. (1997). Assembly of $GABA_A$ receptors composed of α1 and β2 subunits in both cultured neurons and fibroblasts. *J. Neurosci.*, **17**, 6587-6596.

GRAHAM, D., HAURE, C., BESNARD, F. AND LANGER, S.Z. (1996). Pharmacological profile of benzodiazepine site ligands with recombinant $GABA_A$ receptor subtypes. *Eur. Neuropsychopharm.*, **6**, 119-125.

GREEN, W.N. AND CLAUDIO, T. (1993). Acetylcholine receptor assembly: subunit folding and oligomerization occur sequentially. *Cell*, **74**, 57-69.

GREEN, W.N. AND MILLAR, N.S. (1995). Ion-channel assembly. *Trends Neurosci.*, **18**, 280-287.

GREENLEE, D.V. VAN NESS, P.C. AND OLSEN, R.W. (1978a). Gamma-aminobutyric acid binding in mammalian brain: receptor-like specificity of sodium-independent sites. *J. Neurochem.*, **31**, 933-938.

GREENLEE, D.V., VAN NESS, P.C. AND OLSEN, R.W. (1978b). Endogenous inhibitor of GABA binding in mammalian brain. *Life Sci.*, **22**, 1653-1662.

GRIFFON, N., BUTTNER, C., NICKE, A., KUHSE, J., SCHMALZING, G. AND BETZ, H. (1999). Molecular determinants of glycine receptor subunit assembly. *EMBO J.*, **18**, 4711-4721.

GUIDOTTI, A., TOFFANO, G. AND COSTA, E. (1978). An endogenous protein modulates the affinity of GABA and benzodiazepine receptors in rat brain. *Nature*, **275**, 553-555.

GUIDOTTI, A., GALE, K., SURIA, A. AND TOFFANO, G. (1979). Biochemical evidence for two classes of GABA receptors in rat brain. *Brain Res.*, **172**, 566-571.

GURLEY, D.; AMIN, J., ROSS, P.C., WEISS, D.S. AND WHITE, G. (1995). Point mutations in the M2 region of the α, β, or γ subunit of the $GABA_A$ channel that abolish block by picrotoxin. *Recept. Chann.*, **3**, 13-20.

HACKAM, A.S., WANG, T.-L., GUGGINO, W.R. AND CUTTING, G.R. (1997). The N-terminal domain of human GABA receptor ρ1 subunits contains signals for homooligomeric and heterooligomeric interaction. *J. Biol. Chem.*, **272**, 13750-13757.

HADINGHAM, K.L., WINGROVE, P.B., WAFFORD, K.A., BAIN, C., KEMP, J.A., PALMER, K.J., WILSON, A.W., WILCOX, A.S., SIKELA, J.M., RAGAN, C.I. AND WHITING, P.J. (1993). The role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.*, **44**, 1211-1218.

HADINGHAM, K.L., WAFFORD, K.A., THOMPSON, S.A., PALMER, K.J. AND WHITING, P.J. (1995). Expression and pharmacology of human $GABA_A$ receptors containing γ3 subunits. *Eur. J. Pharmacol.*, **291**, 301-309.

HADINGHAM, K.L., GARRETT, E.M., WAFFORD, K.A., BAIN, C., HEAVENS, R.P., SIRINATHSINGHJI, D.J.S. AND WHITING, P.J. (1996). Cloning of cDNAs encoding the human γ-aminobutyric acid type A receptor α6 subunit and characterization of the pharmacology of α6-containing subunits. *Mol. Pharmacol.*, **49**, 253-259.

HAMMOND, C. AND HELENIUS, A. (1995). Quality control in the secretory pathway. *Curr. Opin. Cell Biol.*, **7**, 523-529.

HAMMOND, C., BRAAKMAN, I. AND HELENIUS, A. (1994). Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci., U.S.A.*, **91**, 913-917.

HARVEY, R.J., CHINCHERTRU, M.A. AND DARLISON, M.G. (1994). Alternative splicing of a 51-nucleotide exon that encodes a putative protein kinase c phosphorylation site generates two forms of the chicken γ-aminobutyric $acid_A$ receptor β2 subunit. *J. Neurochem.*, **62**, 10-16.

HAUSER, C.A.E., WETZEL, C.H.R., BERNING, B., GERNER, F.M. AND RUPPRECHT, R. (1997). Flunitrazepam has an inverse agonistic effect on recombinant $\alpha_6\beta_2\gamma_2$-$GABA_A$ receptors via a flunitrazepam-binding site. *J. Biol. Chem.*, **272**,11723-11727.

HEUSCHNIEDER, G. AND SCHWARTZ, R.D. (1989). cAMP and forskolin decrease γ-aminobutryic acid-gated chloride flux in rat brain synaptoneurosomes. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2938-2942.

HEAULME, M., CHAMBON, J.-P., LEYRIS, R., WERMUTH, C.G. AND BIZIERE, K. (1987). Characterization of the binding of [^{3}H]SR95531, a GABA$_A$ antagonist, to rat brain membranes. *J. Neurochem.*, **48**, 1677-1686.

HEIDMANN, T. AND CHANGEUX, J.-P. (1980). Interaction of a fluorescent agonist with the membrane-bound acetylcholine receptor from *Torpedo marmorata* in the millisecond time range: resolution of an 'intermediate' conformational transition and evidence for positive cooperative effects. *Biochem. Biophys. Res. Comm.*, **97**, 889-896.

HELENIUS, A., MARQUARDT, T. AND BRAAKMAN, I. (1992) The endoplasmic reticulum as a protein-folding compartment. *Trends Cell Biol.*, **2**, 227-231.

HERB, A., WISDEN, W., LÜDDENS, H., PUIA, G., VICINI, S. AND SEEBURG, P.H. (1992). The third γ subunit of the γ-aminobutyric acid type A receptor family. *Proc. Natl. Acad. Sci.*, **89**, 1433-1437.

HEVERS, W. AND LÜDDENS, H. (1998). The diversity of GABA$_A$ receptors. *Mol. Neurobiol.*, **18**, 35-86.

HORNSTEIN, J. AND AKABAS, M.H. (1998). Location of a high affinity Zn^{2+} binding site in the channel of α1β1 γ-aminobutyric acid$_A$ receptors. *Mol. Pharmacol.*, **53**, 870-877.

HUGANIR, R.L. AND GREENGARD, P. (1990). Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron*, **5**, 555-567.

HURTLEY, S.A. AND HELENIUS, A. (1989). Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.*, **5**, 277-307.

IM, H.K., IM, W.B., HAMILTON, B.J., CARTER, D.B. AND VONVOIGTLANDER, P.F. (1993a). Potentiation of γ-aminobutyric acid-induced chloride currents by various benzodiazepine site agonists with the α1γ2, β2γ2, and α1β2γ2 subtypes of cloned γ-aminobutyric acid type A receptors. *Mol. Pharmacol.*, **44**, 866-870.

IM, H.K., IM, W.B., JUDGE, T.N., GAMMILL., R.B., HAMILTON, B.J., CARTER, D.B. AND PREGENZER, J.F. (1993b). Substituted pyrazinones, a new class of allosteric modulators for γ-aminobutyric acid$_A$ receptors. *Mol. Pharmacol.*, **44**, 468-472.

IM, W.B., PREGENZER, J.F., BINDER, J.A., DILLON, G.H. AND ALBERTS, G.L. (1995). Chloride channel expression with the tandem construct of α6-β2 GABA$_A$ receptor subunit requires a monomeric subunit of α6 or γ2. *J. Biol. Chem.*, **270**, 26063-26066.

IM, W.B., PREGENZER, J.P., BINDER, J.A. ALBERTS, G.L. AND IM, H.K. (1997). Alterations of the benzodiazepine site of rat α6β2γ2-GABA$_A$ receptor by replacement of several divergent amino-terminal regions with the α1 counterparts. *Br. J. Pharmacol.*, **120**, 559-564.

JACQUES, P, PERRET, O., BOUCHET,M.-J., FOUCAUD, B., GOELDNER, M. AND BENKE, D. (1999). Irreversible site-directed labeling of the 4-aminobutyrate binding site by tritiated *meta*-sulfonate benzene diazonium. *Eur. J. Biochem.*, **264**, 189-194.

JONES, M.V., SAHARA, Y., DZUBAY, J.A. AND WESTBROOK, G.L. (1998). Defining affinity with the GABA$_A$ receptor. *J. Neurosci.*, **18**, 8590-8604.

JOYCE, K.A., ATKINSON, A.E., BERMUDEZ, I., BEADLE, D.J. AND KING, L.A. (1993). Synthesis of functional GABA$_A$ receptors in stable insect cell lines. *FEBS Lett.*, **335**, 61-64.

JURSKY, F., FUCHS, K., BUHR, A., TRETTER, V., SIGEL, E. AND SIEGHART, W. (2000). Identification of amino acid residues of GABA$_A$ receptor subunits contributing to the formation and affinity of the *tert*-butylbicyclophosphorothionate binding site. *J. Neurochem.*, **75**, 1310-1316.

KAO, P.N., DWORK, A.J., KALDANY, R.-R.J., SILVER, M.L., WIDEMAN, J., STEIN, S. AND KARLIN, A. (1984). Identification of the α subunit half-cystine specifically labeled by an affinity reagent for the acetylcholine receptor binding site. *J. Biol. Chem.*, **259**, 11662-11665.

KAO, P.N. AND KARLIN, A. (1986). Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. *J. Biol. Chem.*, **261**, 8085-8088.

KARDOS, J., MADERSPACH, K. AND SIMONYI, M. (1985). Towards a more physiological approach in GABA binding. *Neurochem. Int.*, **7**, 737-743.

KHAN, Z.U., GUTIERREZ, A. AND DE BLAS, A.L. (1994a). The subunit composition of a GABA$_A$/benzodiazepine receptor from rat cerebellum. *J. Neurochem.*, **63**, 371-374.

KHAN, Z.U., GUTIÉRREZ, A. AND DE BLAS, A.L. (1994b). Short and long form γ_2 subunits of the GABA$_A$/benzodiazepine receptors. *J. Neurochem.*, **63**, 1466-1476.

KHAN, Z.U., GUTIÉRREZ, A. AND DE BLAS, A.L. (1996). The α_1 and α_6 subunits can coexist in the same cerebellar GABA$_A$ receptor maintaining their individual benzodiazepine-binding specificities. *J. Neurochem.*, **66**, 685-691.

KHRESTCHATISKY, M., MACLENNAN, A.J., CHIANG, M.-Y., XU, W., JACKSON, M.B., BRESCHA, N., STERNINI, C., OLSEN, R.W. AND TOBIN, A.J. (1989). A novel α subunit in rat brain $GABA_A$ receptors. *Neuron*, **3**, 745-753.

KIRKNESS, E.F., BOVENKERK, C.F., UEDA, T. AND TURNER, A.J. (1989). Phosphorylation of γ-aminobutyrate (GABA)/benzodiazepine receptors by cyclic AMP-dependent protein kinase. *Biochem. J.*, **259**, 613-616.

KIRKNESS, E.F. AND FRASER, C.M. (1993). A strong promoter element is located between alternative exons of a gene encoding the human γ-aminobutyric acid-type A receptor β3 subunit (GABRB3). *J. Biol. Chem.*, **268**, 4420-4428.

KLAUSBERGER, T., FUCHS, K., MAYER, B., EHYA, N. AND SIEGHART, W. (2000). $GABA_A$ receptor assembly: identification and structure of γ_2 sequences forming the intersubunit contacts with α_1 and β_3 subunits. *J. Biol. Chem.*, **275**, 8921-8928.

KLEINGOOR, C., EWERT, M., VON BLAKENFELD, G., SEEBURG, P.H. AND KETTENMANN, H. (1991). Inverse but not full benzodiazepine agonists modulate recombinant $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptors in transfected human embryonic kidney cells. *Neurosci. Lett.*, **130**, 169-172.

KLEINGOOR, C., WIELAND, H.A., KORPI, E.R., SEEBURG, P.H. AND KETTENMANN, H. (1993). Current potentiation by diazepam but not GABA sensitivity is determined by a single histidine residue. *NeuroReport*, **4**, 187-190.

KNOFLACH, F., RHYNER, TH., VILLA, M., KELLENBERGER, S., DRESCHER, U., MALHERBE, P., SIGEL, E. AND MÖHLER, H. (1991). The γ3 subunit of the $GABA_A$-receptor confers sensitivity to benzodiazepine receptor ligands. *FEBS Lett.*, **293**, 191-194.

KOFUJI, P., WANG, J.B., MOSS, S.J., HUGANIR, R.L. AND BURT, D.R. (1991). Generation of two forms of the γ-aminobutyric acid a receptor γ_2-subunit in mice by alternative splicing. *J. Neurochem.*, **56**, 713-715.

KORPI, E.R. AND SEEBURG, P.H. (1993). Natural mutation of $GABA_A$ receptor α6 subunit alters benzodiazepine affinity but not allosteric GABA effects. *Eur. J. Pharmacol. (Mol. Pharmacol. Sect.)*, **247**, 23-27.

KORPI, E.R., KUNER, T., KRISTO, P., KÖHLER, M., HERB, A., LÜDDENS, H. AND SEEBURG, P.H. (1994). Small N-terminal deletion by splicing in cerebellar α6 subunit abolishes $GABA_A$ receptor function. *J. Neurochem.*, **63**, 1167-1170.

KREIENKAMP, H-.J., MAEDA, R.K., SINE, S.M. AND TAYLOR, P. (1995). Intersubunit contacts governing assembly of the mammalian nicotinic acetylcholine receptor. *Neuron*, **14**, 635-644.

KUCKEN, A.M., WAGNER, D.A., WARD, P.R., TEISSÉRE, J.A., BOILEAU, A.J. AND CZAJKOWSKI, C. (2000). Identification of benzodiazepine binding site residues in the γ2 subunit of the γ-aminobutyric $acid_A$ receptor. *Mol. Pharmacol.*, **57**, 932-939.

KUHSE, J., SCHMIEDEN, V., MAGALEI, D., AND BETZ, H. (1990). A single amino acid exchange alters the pharmacology of neonatal glycine receptor subunit. *Neuron*, **5**, 867-873.

KUHSE, J., LAUBE, B., MAGALEI, D. AND BETZ, H. (1993). Assembly of the inhibitory glycine receptor: identification of amino acid sequence motifs governing subunit stoichiometry. *Neuron*, **11**, 1049-1056.

KURIOKA, S., HAYATA, H. AND MATSUDA, M. (1981a). Characterization of γ-aminobutyric acid binding sites on crude synaptic membranes. *J. Neurochem.*, **37**, 283-288.

KURIOKA, S., KIMURA, Y. AND MATSUDA, M. (1981b). Effects of sodium and bicarbonate ions on γ-aminobutyric acid receptor binding in synaptic membranes of rat brain. *J. Neurochem.*, **37**, 418-421.

LAM, D.W. AND REYNOLDS, J.N. (1998). Modulatory and direct effects of propofol on recombinant GABA$_A$ receptors expressed in *Xenopus* oocytes: influence of α and γ2 subunits. *Brain Res.*, **784**, 179-187.

LEACH, M.J. AND WILSON, J.A. (1978). GABA receptor binding with ^{3}H-muscimol in calf cerebellum. *Eur. J. Pharmacol.*, **48**, 329-330.

LEVITAN, E.S, SCHOFIELD, P.R., BURT, D.R., RHEE, L.M., WISDEN, W., KÖHLER, M., FUJITA, N., RODRIGUEZ, H.F., STEPHENSON, A., DARLISON, M.G., BARNARD, E.A. AND SEEBURG, P.H. (1988). Structural and functional basis for GABA$_A$ receptor heterogeneity. *Nature*, **335**, 76-79.

LI,. M. AND DE BLAS, A. (1997). Coexistence of two β subunit isoforms in the same γ-aminobutyric acid type A receptor. *J. Biol. Chem.*, **272**, 16564-16569.

LIN, Y.-F., ANGELOTTI, T.P., DUDEK, E.M., BROWNING, M.D. AND MACDONALD, R.L. (1996). Enhancement of recombinant α1β1γ2L γ-aminobutyric acid$_A$ receptor whole-cell currents by protein kinase C is mediated through phosphorylation of both β1 and γ2L subunits. *Mol. Pharmacol*, **50**, 185-195.

LU, L. AND HUANG, Y. (1998). Separate domains for desensitization of GABA ρ_1 and β_2 subunits expressed in *Xenopus* oocytes. *J. Membr. Biol.*, **164**, 115-124.

LÜDDENS, H., SEEBURG, P.H. AND KORPI, E. (1994). Impact of β and γ variants on ligand-binding properties of γ-aminobutyric acid type A receptors. *Mol. Pharmacol.*, **45**, 810-814.

LÜDDENS, H AND KORPI, E. (1995). GABA antagonists differentiate between recombinant $GABA_A$/benzodiazepine receptor subtypes. *J. Neurosci.*, **15**, 6957-6962.

LÜDDENS, H. AND WISDEN, W. (1991). Function and pharmacology of multiple $GABA_A$ receptor subunits. *Trends Pharmacol. Sci.*, **12**, 49-51.

MADTES JR., P. (1984) Chloride ions preferentially mask high-affinity GABA binding sites. *J. Neurochem.*, **43**, 1434-1437.

MADTES JR., P., BASHIR-ELAHI, R. AND CHADER, G.J. (1986). Maximal GABA and muscimol binding to high-affinity sites differ in physiological and non-physiological buffers. *Neurochem. Int.*, **8**, 223-227.

MADTES JR., P., FULLER, G. AND GREGG, K. (1989). Sensitivity of GABA-displaceable [^{3}H]muscimol binding depends upon the composition of the buffering medium. *Neurochem. Int.*, **15**, 179-183.

MAKSAY, G. (1988). $GABA_A$ receptor populations bind agonists and antagonists differentially and with opposite affinities. *J. Neurochem.*, **50**, 1865-1871.

MAKSAY, G. (1994) Thermodynamics of γ-aminobutyric acid type A receptor binding differentiate agonists from antagonists. *Mol. Pharmacol.*, **46**, 386-390.

MAKSAY, G. (1998). Differential effects of thiocyanate on the binding thermodynamics of bicuculline methiodide versus SR95531 (gabazine) to the γ-aminobutyric acid receptor-ionophore complex. *Biochem. Pharmacol.*, **56**, 729-731.

MAKSAY, G. and TICKU, M.K. (1984a). Characterization of γ-aminobutyric acid-benzodiazepine receptor complexes by protection against inactivation by group-specific reagents. *J. Neurochem.*, **42**, 1715-1727.

MAKSAY, G. AND TICKU, M.K. (1984b). Diazotization and thiocyanate differentiate agonists from antagonists for the high- and low-affinity receptors of γ-aminobutyric acid. *J. Neurochem.*, **43**, 261-268.

MALHERBE, P., SIGEL, E., BAUR, R., RICHARDS, J.G. AND MOHLER, H. (1990). Functional expression and sites of gene transcription of a novel α subunit of the $GABA_A$ receptor in rat brain. *FEBS Lett.*, **260**, 261-265.

MARANGOS, P.J. AND MARTINO, A.M. (1981). Studies on the relationship of γ-aminobutyric acid-stimulated diazepam binding and the γ-aminobutyric acid receptor. *Mol. Pharmacol.*, **20**, 16-21.

MARTIN, M., CZAJKOWSKI, C. AND KARLIN, A. (1996). The contributions of aspartyl residues in the acetylcholine receptor γ and δ subunits to the binding of agonists and competitive antagonists. *J. Biol. Chem.*, **271**, 13497-13503.

MATSUMOTO, K. AND FUKUDA, H. (1982). Stimulatory and protective effects of benzodiazepines on GABA receptors labeled with [^{3}H]muscimol. *Life Sci.*, **30**, 935-943.

MCCABE, R.T., WAMSLEY, J.K., YEZUITA, J.P. AND OLSEN, R.W. (1988). A novel $GABA_A$ antagonist [^{3}H]SR95531: microscopic analysis of binding in the rat brain and allosteric modulation by several benzodiazepine and barbiturate receptor ligands. *Synapse*, **2**, 163-173.

MCKERNAN, R.M. AND WHITING, P.J. (1996). Which $GABA_A$-receptor subtypes really occur in the brain? *Trends Neurosci.*, **19**, 139-143

MEINERS, B.A. AND SALAMA, A.I. (1985). Enhancement of GABA binding by the benzodiazepine partial agonist CGS9896. *Eur. J. Pharmacol.*, **119**, 61-65.

MERTENS, S., BENKE, D. AND MÖHLER, H. (1993). $GABA_A$ receptor populations with novel subunit combinations and drug binding profiles identified in brain by α5- and δ-subunit-specific immunopurification. *J. Biol. Chem.*, **268**, 5965-5973.

MICHELSON, H.B. AND WONG, R.K.S. (1991). Excitatory synaptic responses mediated by $GABA_A$ receptors in the hippocampus. *Science*, **253**, 1420-1423.

MIDDLETON, R.E. AND COHEN, J.B. (1991). Mapping of the acetylcholine binding site of the nicotinic acetylcholine receptor: [^{3}H]nicotine as an agonist photoaffinity label. *Biochemistry*, **30**, 6987-6997.

MIHIC, S.J., WHITING, P.J., KLEIN, R.L., WAFFORD, K.A. AND HARRIS, R.A. (1994). A single amino acid of the human γ-aminobutyric acid type A receptor γ_2 subunit determines benzodiazepine efficacy. *J. Biol. Chem.*, **269**, 32768-32773.

MÖHLER, H. AND OKADA, T. (1978). Properties of γ-aminobutyric acid receptor binding with (+)- [^{3}H]bicuculline methiodide in rat cerebellum. *Mol. Pharmacol.*, **14**, 256-265.

MONOD, J., WYMAN, J. AND CHANGEUX, J.-P., (1965). On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.*, **12**, 88-118.

MOSS, S.J., SMART, T.G., BLACKSTONE, C.D. AND HUGANIR, R.L. (1992). Functional modulation of $GABA_A$ receptors by cAMP-dependent protein phosphorylation. *Science*, **257**, 661-665.

MOSS, S.J., GORRIE. G.H., AMATO, A. AND SMART, T.G. (1995). Modulation of $GABA_A$ receptors by tyrosine phosphorylation. *Nature*, **377**, 344-348.

NAYEEM, N., GREEN, T.P., MARTIN, I.L. AND BARNARD, E.A. (1994). Quaternary structure of the native $GABA_A$ receptor determined by electron microscopic image analysis. *J. Neurochem.*, **62**, 815-818.

NEWELL, J.G., DAVIES, M., BATESON, A.N. AND DUNN, S.M.J. (2000). Tyrosine 62 of the γ-aminobutyric acid type A receptor β2 subunit is an important determinant of high affinity agonist binding. *J. Biol. Chem.*, **275**, 14198-14204.

NIELSEN, M. AND BRAESTRUP, C. (1980). Ethyl β-carboline-3-carboxylate shows differential benzodiazepine receptor interaction. *Nature*, **286**, 606-607.

O'LEARY, M.E AND WHITE, M.M. (1992). Mutational analysis of ligand-induced activation of the *Torpedo* acetylcholine receptor. *J. Biol. Chem.*, **267**, 8360-8365.

O'LEARY, M.E., FILATOV, G.N. AND WHITE, M.M. (1994) Characterization of *d*-tubocurarine binding site of *Torpedo* nicotinic acetylcholine receptor. *Am. J. Physiol.*, **266**, C648-653.

O'SHEA, S. AND HARRISON, N.L. (2000). Propofol increases agonist efficacy at the $GABA_A$ receptor. *Brain Res.*, **852**, 344-348.

OLSEN, R.W., BERGMAN, M.O., VAN NESS, P.C., LUMMIS, S.C., WATKINS, A.E., NAPIAS, C. AND GREENLEE, D.V. (1981). γ-Aminobutyric acid receptor binding in mammalian brain: heterogeneity of binding sites. *Mol. Pharmacol.*, **19**, 217-227.

OLSEN, R.W. AND SNOWMAN, A.M. (1982). [^{3}H]Bicuculline methochloride binding to low-affinity γ-aminobutyric acid receptor sites. *J. Neurochem.*, **41**, 1653-1663.

OLSEN, R.W., YANG, J., KING, R.G., DILBER, A., STAUBER, G.B. AND RANSOM, R.W. (1986). Benzodiazepine receptor function: V. barbiturate and benzodiazepine modulation of GABA receptor binding and function. *Life Sci.*, **39**, 1969-1976.

OU, W.-J., CAMERSON, P.H., THOMAS, D.Y. AND BERGERON, J.J.M. (1993). Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature*, **364**, 771-776.

PÁLDI-HARIS, P., GRÁF, L., KENESSY, Á. AND LÁNG, T. (1985). Enhancement of low affinity [^{3}H]GABA binding by diazepam and a new 2,3 benzodiazepine. *Eur. J. Pharmacol.*, **109**, 305-306.

PEDERSEN, S.E. AND COHEN, J.B. (1990). *d*-Tubocurarine binding sites are located at the α-γ and α-δ subunit interfaces of the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci.*, **87**, 2785-2789.

PELHAM, H.R.B. (1989). Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.*, **5**, 1-23.

POLLARD, S., DUGGAN, M.J. AND STEPHENSON, F.A. (1993). Further evidence for the existence of α subunit heterogeneity within discrete γ-aminobutyric acid$_A$ receptor sub-populations. *J. Biol. Chem.*, **268**, 3753-3757.

PREGENZER, J.F., IM, W.B., CARTER, D.B. AND THOMSEN, D.R. (1993). Comparison of interactions of [^{3}H]muscimol, *t*-butylbicyclophosphoro[^{35}S]thionate and [^{3}H]flunitrazepam with cloned γ-aminobutyric acid$_A$ receptors of the $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ subtypes. *Mol. Pharmacol.*, **43**, 801-806.

PRINCE, R.J. AND SINE, S.M. (1996). Molecular dissection of subunit interfaces in the acetylcholine receptors: identification of residues that determine agonist selectivity. *J. Biol. Chem.*, **271**, 25770-25777.

PRITCHETT, D.B., SONTHEIMER, H., GORMAR, C.M., KETTENMANN, H., SEEBURG, P.H. AND SCHOFIELD, P.R. (1988). Transient expression shows ligand gating and allosteric potentiation of $GABA_A$ receptor subunits. *Science*, **242**, 1306-1308.

PRITCHETT, D.B., SONTHEIMER, H., SHIVERS, B.D., YMER, S., KETTENMANN, H., SCHOFIELD, P.R. AND SEEBURG P.H. (1989a). Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature*, **338**, 582-585.

PRITCHETT, D.B., LÜDDENS, H. AND SEEBURG, P.H. (1989b). Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science*, **245**, 1389-1392.

PRITCHETT, D.B. AND SEEBURG, P.H. (1990). γ-Aminobutyric $acid_A$ receptor α_5-subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.*, **54**, 1802-1804.

PRITCHETT, D.B. AND SEEBURG, P.H. (1991). γ-Aminobutyric acid type A receptor point mutation increases the affinity for compounds for the benzodiazepine site. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 1421-1425.

PUIA, G., VICINI, S., SEEBURG, P.H. AND COSTA, E. (1991). Influence of recombinant γ-aminobutyric $acid\text{-}_A$ receptor subunit composition on the action of allosteric modulators of γ-aminobutyric acid-gated Cl^- currents. *Mol. Pharmacol.*, **39**, 691-696.

PUIA, G., SANTI., M., VICINI., S., PRITCHETT, D.B., PURDY, R.H., PAUL, S.M., SEEBURG, P.H. AND COSTA, E. (1990). Neurosteroids act on recombinant $GABA_AR$. *Neuron*, **4**, 759-765.

QUIRK, K., GILLARD, N.P., RAGAN, C.I., WHITING, P.J. AND MCKERNAN, R.M. (1994). γ-Aminobutyric acid type A receptors in the rat brain can contain both γ2 and γ3 subunits, but γ1 does not exist in combination with another γ subunit. *Mol. Pharmacol.*, **45**, 1061-1070.

RAFTERY, M.A., DUNN, S.M.J., CONTI-TRONCONI, B.M., MIDDLEMAS, D.S. AND CRAWFORD, R.D. (1983). The nicotinic acetylcholine receptor: subunit structure, functional binding sites, and ion transport properties. *Cold Spring Harb. Symp. Quant. Biol.*, **48**, 21-33.

RAJENDRA, S., VANDENBERG, R.J., PIERCE, K.D., CUNNINGHAM, A.M., FRENCH, P.W., BARRY, P.H. AND SCHOFIELD, P.R. (1995). The unique extracellular disulfide loop of the glycine receptor is a principal ligand binding element. *EMBO J.*, **14**, 2987-2998.

RENARD, S., OLIVIER, A., GRANGER, P., AVENET, P., GRAHAM, D., SEVRIN, M., GEORGE, P. AND BESNARD, F. (1999). Structural elements of the γ-aminobutyric acid type A receptor conferring subtype selectivity for benzodiazepine site ligands. *J. Biol. Chem.*, **274**, 13370-13374.

ROBERTS, E. AND SHERMAN, M.A. (1992). GABA-the quintessential neurotransmitter: electroneutrality, fidelity, specificity, and a model for the ligand binding site of $GABA_A$ receptors. *Neurochem. Res.*, **18**, 365-376.

ROSS, A.F., GREEN, W.N., HARTMAN, D.S. AND CLAUDIO, T. (1991). Efficiency of acetylcholine receptor subunit assembly and its regulation by cAMP. *J. Cell Biol.*, **113**, 623-636.

RUIZ-GOMEZ, A., MORATO, E., GARCIÁ-CALVO, M., VALDIVIESO, F. AND MAYOR JR., F. (1990). Localization of the strychnine binding site on the 48-kilodalton subunit of the glycine receptor. *Biochemistry*, **29**, 7033-7040.

SAEDI, M.S., CONROY, W.G. AND LINDSTROM, J. (1991). Assembly of *Torpedo* acetylcholine receptors in *Xenopus* oocytes. *J. Cell Biol.*, **112**, 1007-1015.

SCHAERER, M.T., BUHR, A., BAUR, R. AND SIGEL, E. (1998). Amino acid residue 200 on the α_1 subunit of $GABA_A$ receptors affects the interaction with selected benzodiazepine binding site ligands. *Eur. J. Pharmacol.*, **254**, 283-287.

SCHMIEDEN, V., KUHSE, J. AND BETZ, H. (1999). A novel domain of the inhibitory glycine receptor determining antagonist efficacies: further evidence for partial agonism resulting from self-inhibition. *Mol. Pharmacol.*, **56**, 464-472.

SCHOFIELD, P.R., DARLISON, M.G., FUJITA, N., BURT, D.R., STEPHENSON, F.A., RODRIGUEZ, H., RHEE, L.M., RAMACHANDRAN, J., REALE, V., GLENCORSE, T.A., SEEBURG, P.H. AND BARNARD, E.A. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature*, **328**, 221-227.

SCHOLZE, P., EBERT, V. AND SIEGHART, W. (1996). Affinity of various ligands for $GABA_A$ receptors containing $\alpha_4\beta_3\gamma_2$, $\alpha_4\gamma_2$, or $\alpha_1\beta_3\gamma_2$ subunits. *Eur. J. Pharmacol.*, **304**, 155-162.

SEEBURG, P.H., WISDEN, W., VERDOORN, T.A., PRITCHETT, D.B., WERNER, P., HERB, A., LÜDDENS, H., SPRENGEL, R. AND SAKMANN, B. (1990). The $GABA_A$ receptor family: molecular and functional diversity. *Cold Harb. Sym. Quant. Biol.*, **40**, 29-40.

SHANK, R.P., BALDY, W.J., MATTUCCI, L.C. AND VILLANI, JR. F.J. (1990). Ion and temperature effects on the binding of γ–aminobutyrate to its receptors and the high-affinity transport system. *J. Neurochem.*, **54**, 2007-2015.

SIEGHART, W. (1989). Multiplicity of $GABA_A$-benzodiazepine receptors. *Trends Pharmacol. Sci.*, **10**, 407-411.

SIEGHART, W. (1995). Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharmacol.Rev.*, **47**, 181-234.

SIGEL, E., STEPHENSON, F.A., MAMALAKI, C. AND BARNARD, E.A. (1983). A γ-aminobutyric acid/benzodiazepine receptor complex of bovine cerebral cortex: purification and partial characterization. *J. Biol. Chem.*, **258**, 6965-6971.

SIGEL, E., AND BARNARD, E.A. (1984). A γ-aminobutyric acid/benzodiazepine receptor complex from bovine cerebral cortex: improved purification with preservation of regulatory sites and their interactions. *J. Biol. Chem.*, **259**, 7219-7223.

SIGEL, E., AND BAUR, R. (1988). Activation of protein kinase C differentially modulates neuronal Na^+, Ca^{2+}, and γ-aminobutyric acid type A channels. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 6192-6196.

SIGEL, E., BAUR, R., TRUBE, G., MÖHLER, H. AND MALHERBE, P. (1990). The effect of subunit composition of rat brain $GABA_A$ receptor on channel function. *Neuron*, **5**, 703-711.

SIGEL, E., BAUR, R., KELLENBERGER, S. AND MALHERBE, P. (1992). Point mutations affecting antagonist and agonist dependent gating of $GABA_A$ receptor channels. *EMBO J.*, **11**, 2017-2023.

SIGEL, E. AND BUHR, A. (1997). The benzodiazepine binding site of $GABA_A$ receptors. *Trends Pharmacol. Sci.*, **18**, 425-429.

SIGEL, E., SCHAERER, M.T., BUHR, A. AND BAUR, R. (1997). The benzodiazepine binding pocket of recombinant α1β2γ2 γ-aminobutyric acid$_A$ receptors: relative orientation of ligands and amino acid side chains. *Mol. Pharmacol.*, **54**, 1097-1105.

SIGEL E., BUHR, A. AND BAUR, R. (1999). Role of the conserved lysine residue in the middle of the predicted extracellular loop between M2 and M3 in the GABA$_A$ receptor. *J. Neurochem.*, **73**, 1758-1764.

SIGEL, E., AND BAUR, R. (2000). Electrophysiological evidence for the coexistence of α1 and α6 subunits in a single functional GABA$_A$ receptor. *J. Neurochem.*, **74**, 2590-2596.

SINE, S.M. AND CLAUDIO, T. (1991). γ- and δ-Subunits regulate the affinity and the cooperativity of ligand binding to the acetylcholine receptor. *J. Biol. Chem.*, **266**, 19369-19377.

SINE, S.M., QUIRAM, P., PAPANIKOLAOU, F., KREIENKAMP, H.-J. AND TAYLOR, P. (1994). Conserved tyrosines in the α subunit of the nicotinic acetylcholine receptor stabilize quaternary ammonium groups of agonists and curariform antagonists. *J. Biol. Chem.*, **269**, 8808-8816.

SINE, S.M., KREIENKAMP, H.-J., BREN, N., MAEDA, R. AND TAYLOR, P. (1995). Molecular dissection of subunit interfaces in the acetylcholine receptor: identification of determinants of α-conotoxin M-1 selectivity. *Neuron*, **15**, 205-211.

SKERRITT, J.H. AND JOHNSTON, G.A.R. (1983a) Enhancement of GABA binding by benzodiazepines. *Eur. J. Pharmacol.*, **89**, 193-198.

SKERRITT, J.H. AND JOHNSTON, G.A.R. (1983b) Diazepam stimulates the binding of GABA and muscimol but not THIP to rat brain membranes. *Neurosci. Lett.*, **38**, 315-320.

SKERRITT, J.H. AND MACDONALD, R.L. (1984). Benzodiazepine receptor ligand actions on GABA responses: β-carbolines, purines. *Eur. J. Pharmacol.*, **101**, 135-141.

SKERRITT, J.H., CHEN CHOW, S. AND JOHNSTON, G.A.R. (1982a). Differences in the interactions between GABA and benzodiazepine binding sites. *Neurosci. Lett.*, **33**, 173-178.

SKERRITT, J.H., WILLOW, M. AND JOHNSTON, G.A.R. (1982b). Diazepam enhancement of low affinity GABA binding to rat brain membranes. *Neurosci. Lett.*, **29**, 63-66.

SMART, T.G., MOSS, S.J., XIE, X. AND HUGANIR, R.L. (1991). GABA$_A$ receptors are differentially sensitive to zinc dependence on subunit compositions. *Br. J. Pharmacol.*, **103**, 1837-1839.

SMITH, G.B. AND OLSEN, R.W. (1994). Identification of [^{3}H]muscimol photoaffinity substrate in the bovine γ-aminobutyric acid$_A$ receptor α subunit. *J. Biol. Chem.*, **269**, 20380-20387.

SMITH, G.B. AND OLSEN, R.W. (2000). Deduction of amino acid residues in the GABA$_A$ receptor α subunit photoaffinity labeled with the benzodiazepine flunitrazepam. *Neuropharmacology*, **39**, 55-64.

SPIER, A.D. AND LUMMIS, S.C.R. (2000). The role of tryptophan residues in the 5-hydroxytryptamine$_3$ receptor ligand binding domain. *J. Biol. Chem.*, **275**, 5620-5625.

SQUIRES, R.F., BENSON, D.J., BRAESTRUP, C., COUPET, J., KLEPNER, C.A., MYERS, V. AND BEER, B. (1979). Some properties of brain specific benzodiazepine receptors: new evidence for multiple receptors. *Pharmacol. Biochem. Behav.*, **10**, 825-830.

SRINIVASAN, S., NICHOLS, C.J., LAWLESS, G.M., OLSEN, R.W. AND TOBIN, A.J. (1999). Two invariant tryptophans on the $\alpha 1$ subunit define domains necessary for $GABA_A$ receptor assembly. *J. Biol. Chem.*, **274**, 26633-26638.

STEPHENSON, F.A. (1988). Understanding the $GABA_A$ receptor: a chemically gated ion channel. *Biochem. J.*, **249**, 21-32.

STEPHENSON, F.A. (1995). The $GABA_A$ receptors. *Biochem. J.*, **310**, 1-9.

STEPHENSON, F.A. AND DUGGAN, M.J. (1989). Mapping the benzodiazepine photoaffinity-labelling site with sequence–specific γ-aminobutyric $acid_A$-receptor antibodies. *Biochem. J.*, **264**, 199-206.

STEPHENSON, F.A., DUGGAN, M.J. AND POLLARD, S. (1990). The γ_2 subunit is an integral component of the γ-aminobutyric acid A receptor but the α_1 polypeptide is the principal site of the agonist benzodiazepine photoaffinity labeling reaction. *J. Biol. Chem.*, **265**, 21160-21165.

STEWARD, L.J., BOESS, F.G., STEELE, J.A., LIU, D., WONG, N. AND MARTIN, I.L. (2000). Importance of phenylalanine 107 in agonist recognition by the 5-$hydroxytryptamine_{3A}$ receptor. *Mol. Pharmacol.*, **57**, 1249-1256.

SULLIVAN, D. AND COHEN, J.B. (2000). Mapping the agonist binding site of the nicotinic acetylcholine receptor: orientation requirements for activation by covalent agonist. *J. Biol. Chem.*, **275**, 12651-12660.

SWEETNAM, P.M., LLOYD, J., GALLOMBARDO, P., MALISON, R.T., GALLAGER, D.W., TALLMAN, J.F. AND NESTLER, E.J. (1988). Phosphorylation of the $GABA_a$/benzodiazpine α subunit by a receptor–associated protein kinase. *J. Neurochem*, **51**, 1274-1284.

TALLMAN, J.F., THOMAS, J.W. AND GALLAGER, D.W. (1978). GABAergic modulation of benzodiazepine site sensitivity. *Nature*, **274**, 383-385.

TAYLOR, P.M., THOMAS, P., GORRIE, G.H., CONNOLLY, C.N., SMART, T.G. AND MOSS, S.J. (1999). Identification of amino acid residues within GABA$_A$ receptor β subunits that mediate both homomeric and heteromeric receptor expression. *J. Neurosci.*, **19**, 6360-6371.

TAYLOR, P.M., CONNOLLY, C.N., KITTLER, J.T., GORRIE, G.H., HOSIE, A., SMART, T.G. AND MOSS, S.J. (2000). Identification of residues within GABA$_A$ receptor α subunits that mediate specific assembly with receptor β subunits. *J. Neurosci.*, **20**,1297-1306.

TEHRANI. M.H.J., HABLITZ, J. AND BARNES, E.M. JR. (1989). cAMP increases the rate of GABA$_A$ receptor desensitization in chick cortical neurons. *Synapse*, **4**, 126-131.

TEISSÉRE, J.A., BOILEAU, A.J. AND CZAJKOWSKI, C (1999). Elucidating the structure of the benzodiazepine binding site on the GABA$_A$ receptor by engineered sulfhydryl modification. *Society for Neuroscience Abstracts.*, **25**, 964.

TIERNEY, M., BIRNIR, B., PILLAI, N.P., CLEMENTS, J.D., HOWITT, S.M., COX, G.B. AND GAGE, P.W. (1996). Effects of mutating leucine to threonine in the M2 segment of α_1 and β_1 subunits of GABA$_A$ $\alpha_1\beta_1$ receptors. *J. Membr. Biol.*, **154**, 11-21.

TRETTER, V., EHYA, N., FUCHS, K. AND SIEGHART, W. (1997). Stoichiometry and assembly of a recombinant GABA$_A$ receptor subtype. *J. Neurosci.*, **17**, 2728-2737.

TURNER, D.M., SAPP, D.W. AND OLSEN, R.W. (1991). The benzodiazepine/alcohol antagonist Ro15-4513: binding to a GABA$_A$ receptor subtype that is insensitive to diazepam. *J. Pharm. Exp. Ther.*, **257**, 1236-1242.

UENO, S., WICK, M.J., YE, Q., HARRISON, N.L. AND HARRIS, R.A. (1999). Subunit mutations affect ethanol actions on $GABA_A$ receptors expressed in *Xenopus* oocytes. *Br. J. Pharmacol.*, **127**, 377-382.

UNWIN, N. (1995). Acetylcholine receptor channel imaged in the open state. *Nature*, **373**, 37-43.

VAFA, B., LEWIS, T.M., CUNNINGHAM, A.M., JACQUES, P., LYNCH, J.W. AND SCHOFIELD, P.R. (1999). Identification of a new ligand binding domain in the α1 subunit of the inhibitory glycine receptor. *J. Neurochem.*, **73**, 2158-2166.

VANDENBERG, R.J., FRENCH, C.R., BARRY, P.H., SHINE, J. AND SCHOFIELD, P.R. (1992a). Antagonism of ligand-gated ion channel receptors: two domains of the glycine receptor α subunit form the strychnine binding site. *Proc. Natl. Acad. Sci.*, **89**, 1765-1769.

VANDENBERG, R.J., HANDFORD, C.A. AND SCHOFIELD, P.R. (1992b). Distinct agonist- and antagonist-binding sites on the glycine receptor. *Neuron*, **9**, 491-496.

VANDENBERG, R.J., RAJENDRA, S., FRENCH, C.R., BARRY, P.H. AND SCHOFIELD, P.R. (1993). The extracellular disulfide loop motif of the inhibitory glycine receptor does not form the agonist binding site. *Mol. Pharmacol.*, **44**, 198-203.

VERRALL, S. AND HALL, Z.W. (1992). The N-terminal domains of acetylcholine receptor subunits contain recognition signals for the initial steps of receptor assembly. *Cell*, **68**, 23-31.

VERDOORN, T.A., DRAUGHN, A., YMER, S., SEEBURG, P.H. AND SAKMANN, B. (1990). Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. *Neuron*, **4**, 919-928.

WAFFORD, K.A., BAIN, C.J., WHITING, P.J. AND KEMP, J.A. (1993). Functional comparison of the role of γ subunits in recombinant human γ-aminobutyric $acid_A$/benzodiazepine receptors. *Mol. Pharmacol.*, **44**, 437-442.

WAFFORD, K.A., THOMPSON, S.A., THOMAS, D., SIKELA, J., WILCOX, A.S. AND WHITING, P.J. (1996). Functional characterization of human γ-aminobutyric $acid_A$ receptors containing the $\alpha4$ subunit. *Mol. Pharmacol.*, **50**, 670-678.

WANG, Y.-J., SALVATERRA, P. AND ROBERTS, E. (1979). Characterization of [^{3}H]muscimol binding to mouse brain membranes. *Biochem. Pharmacol.*, **28**, 1123-1128.

WANG, H., BEDFORD, F.K., BRANDON, N.J., MOSS, S.J. AND OLSEN, R.W. (1999). $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton. *Nature*, **397**, 69-72.

WESTH-HANSEN, S.E., RASMUSSEN, P.B., HASTRUP, S., NABEJURA, J., NOGUCHI, K., AKAIKE, N., WITT, M.-R. AND NIELSEN, M. (1997). Decreased agonist sensitivity of human $GABA_A$ receptors by an amino acid variant, isoleucine to valine, in the $\alpha1$ subunit. *Eur. J. Pharmacol.*, **329**, 253-257.

WESTH-HANSEN, S.E., WITT, M.R., DEKERMENDJIAN, K., LILJEFORS, T., RASMUSSEN, P.B. AND NIELSEN, M. (1999). Arginine residue 120 of the human $GABA_A$ receptor α_1, subunit is essential for GABA binding and chloride ion current gating. *NeuroReport*, **10**, 2417-2421.

WHITE, G., GURLEY, D, HARTNETT, C., STIRLING, V. AND GREGORY, J. (1995). Human α and β subunits contribute to the EC50 for GABA at the $GABA_A$ receptor expressed in Xenopus oocytes. *Recept. Chann.*, **3**, 1-5.

WHITING, P.J., MCKERNAN, R.M. AND IVERSEN, L.I. (1990). Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 9966-9970.

WIELAND, H.A., LÜDDENS, H. AND SEEBURG, P.H. (1992). A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J. Biol. Chem.*, **267**, 1426-1429.

WIELAND, H. AND LÜDDENS, H. (1994). Four amino acid exchanges convert a diazepam-insensitive, inverse agonist-preferring $GABA_A$ receptor into a diazepam-preferring $GABA_A$ receptor. *J. Med. Chem.*, **37**, 4576-4580.

WILLIAMS, M. AND RISLEY, E.A. (1979). Characterization of the binding of [^{3}H]muscimol, a potent γ-aminobutyric acid agonist, to rat brain synaptosomal membranes using a filtration assay. *J. Neurochem.*, **32**, 713-718.

WINGROVE, P.B., THOMPSON, S.A., WAFFORD, K.A. AND WHITING, P.J. (1997) Key amino acids in the γ subunit of the γ-aminobutyric $acid_A$ receptor determine ligand binding and modulation at the benzodiazepine site. *Mol. Pharmacol.*, **52**, 874-881.

XU, M. AND AKABAS, M.H. (1996). Identification of channel-lining residues in the M2 membrane-spanning segment of the $GABA_A$ receptor α_1 subunit. *J. Gen. Physiol.*, **107**, 195-205.

YAN, D., SCHULTE, M.K., BLOOM, K.E. AND WHITE, M.W. (1999). Structural features of the ligand-binding domain of the serotonin $5HT_3$ receptor. *J. Biol. Chem.*, **274**, 5537-5541.

YANG, J.S.-J. AND OLSEN, R.W. (1987). γ-Aminobutyric acid receptor binding in fresh mouse brain membranes at 22°C: ligand-induced changes in affinity. *Mol. Pharmacol.*, **32**, 266-277.

YANG, S.-H., ARMSON, P.F., CHA, J. AND PHILLIPS, W.D. (1997). Clustering of $GABA_A$ receptors by rapsyn/43kD protein *in vitro*. *Mol. Cell. Neurosci.*, **8**, 430-438.

YMER, S, DRAUGHN, A., WISDEN, W., WERNER, P., KEINÄNEN, SCHOFIELD, P.R., SPREGNEL, R., PRITCHETT, D.B. AND SEEBURG, P.H. (1990). Structural and functional characterization of the γ_1 subunit of $GABA_A$/benzodiazepine receptors. *EMBO J.*, **9**, 3261-3267.

ZEZULA, J., SLANY, A. AND SIEGHART, W. (1996). Interaction of allosteric ligands with $GABA_A$ receptors containing one, two, or three different subunits. *Eur. J. Pharmacol.*, **301**, 207-214.

CHAPTER 2[1]

Tyrosine 62 Of The γ-Aminobutyric Acid Type A Receptor β2 Subunit Is An Important Determinant Of High Affinity Agonist Binding

---

[1] *A version of this chapter has been published.* NEWELL, J.G., DAVIES, M., BATESON, A.N., AND DUNN, S.M.J. (2000). *J. Biol. Chem.*, **275**, 14198-14204.

INTRODUCTION

The type A γ-aminobutyric acid receptor ($GABA_AR$) is a member of a superfamily of ligand-gated ion channels (LGIC) that includes the nicotinic acetylcholine receptor (nAChR), the glycine receptor (GlyR) and the serotonin type 3 receptor ($5HT_3R$) (Dunn *et al.*, 1994). The $GABA_AR$ carries binding sites for a number of therapeutic agents including the benzodiazepines, barbiturates, neurosteroids, some general anaesthetics, and possibly also alcohol (Dunn *et al.*, 1994). In brain membranes, there are at least two classes of binding sites for the endogenous neurotransmitter, which differ by more than an order of magnitude in their affinity for GABA or its structural analogues (Olsen *et al.*, 1981; Olsen *et al.*, 1986; Yang and Olsen, 1987; Agey and Dunn, 1989). This heterogeneity in binding was originally thought to reflect the diversity of $GABA_AR$ subtypes in brain tissue. However, the presence of both classes of sites in a stable cell line expressing a specific subtype (Davies *et al.*, 1994) suggests that both exist in a single receptor molecule. On the basis of biochemical studies, the reasonable correlation between the concentration of agonist required to elicit ion flux and to potentiate the binding of benzodiazepine ligands suggested that the low affinity sites are important for channel gating (*see* Sieghart, 1995). However, the role(s) of the high affinity binding sites in receptor function remains unclear.

All members of this receptor family are believed to be pentameric complexes formed by homologous subunits assembled to form a central ion channel (Nayeem *et al.*, 1994). Recent models (*see* Changeux and Edelstein, 1998) predict that ligand binding sites occur at subunit-subunit interfaces. This was first demonstrated in the nAChR in which the α−γ and α−δ interfaces were implicated in forming non-equivalent binding sites for d-tubocurarine (Pedersen and Cohen, 1990). In the $GABA_AR$, low affinity GABA sites (i.e. those that have been implicated in channel activation) are thought to be located at the interfaces between β and α subunits (Sigel *et al.*, 1992; Amin and Weiss, 1993; Smith and Olsen, 1994; Boileau *et al.*, 1999), while the benzodiazepine binding site is predicted to occur at the homologous α−γ interface (Wieland *et al.*, 1992; Buhr *et al.*, 1996; Davies *et al.*, 1996; Duncalfe *et al.*, 1996; Amin *et al.*, 1997; Buhr *et al.*, 1997a; Buhr *et al.*, 1997b). More detailed analyses of the properties of these sites have led to a "loop model" of ligand binding sites (*see* Corringer *et al.*, 2000) in which amino acid residues from at least three discontinuous regions (denoted "loops" A-C) of one subunit together with residues from at least one region of the adjacent subunit ("loop" D) form the binding pocket (Fig. 2-1).

In the GABA$_A$R, evidence for the location of the high affinity agonist site(s) is derived from a number of experimental approaches. Photoaffinity labeling studies first suggested that the β subunit is a major determinant of high affinity binding since this was the principle site of photoincorporation of [^{3}H]muscimol (Casalotti *et al.*, 1986; Deng *et al.*, 1986), although another report has given some indication that the α subunit can also be labelled (Smith and Olsen, 1994). Heterologous expression of different GABA$_A$R subunit combinations indicates that coexpression of α and β subunits is required for high affinity binding and the α1β3, α1β3γ2, α1β2 and α1β2γ2 combinations have all been shown to form high affinity binding sites for [^{3}H]muscimol (Pregenzer *et al.*, 1993; Zezula *et al.*, 1996). Furthermore, expression of a tandem construct in which the C-terminus of α6 was covalently linked to the N-terminus of the β2 subunit produced high affinity binding sites (Im *et al.*, 1995) although the receptors were non-functional unless an additional α6 or γ2 subunit was coexpressed with these constructs.

Based on the above observations and homology considerations, we speculated that a high affinity agonist site in the GABA$_A$R may be located at the α−β subunit interface, in which the β subunit would contribute residues in "loop D" according to the model described above (Fig. 2-1). Candidate tyrosine residues (at positions 62 and 74) of the β2 subunit were identified by amino acid sequence alignment (Fig. 2-2) based on previous work that residues in the homologous positions to Y62 in the α and γ subunits have been implicated in (low affinity) GABA and benzodiazepine binding, respectively (Sigel *et al.*, 1992; Smith and Olsen, 1994; Buhr *et al.*, 1997a,b). Both tyrosine residues were mutated to phenylalanine and to serine in order to evaluate the relative contributions of the aromatic rings and hydroxyl groups of these tyrosine residues to high affinity muscimol binding.

In this chapter, I demonstrate that Y62 of the β subunit is an important determinant of high affinity muscimol binding. Substitution of phenylalanine at this position decreased the affinity for both mucimol and GABA whereas substitution by serine led to a loss of detectable high affinity binding sites. In functional assays, both mutations increased the EC_{50} for channel activation. These results suggest that Y62 of the β subunit is an important determinant for high affinity agonist binding and that, although this residue may play some role in receptor activation, high affinity binding *per se* is not a requirement for channel gating.

MATERIALS AND METHODS

Site-Directed Mutagenesis

The β2 mutants were generated using the Altered Sites® II *in vitro* Mutagenesis System from the Promega Corporation (Madison, WI, USA). The protocol for this system has been described (Newell *et al.*, 2000). Single point mutants are named according to their position in the mature β2 subunit (Fig. 2-2), which was determined by the calculation of the signal peptide cleavage site according to the algorithm of Nielsen *et al.* (1997). The following oligonucleotides were designed for the mutagenesis procedure and were purified by polyacrylamide gel electrophoresis according to the method previously described (Tanay *et al.*, 1997):

Y62F, 5' TACACCTTGACCATGTTTTTCCAGCAAGCTTGGAGAGATAAGAGA 3'
Y62S, 5' TACACCTTGACCATGTCTTTCCAGCAAGCTTGGAGAGATAAGAGA 3'
Y74F, 5' TATTTCCAGCAAGCTTGGAGAGATAAGAGACTGTCCTTCAATGTAATCCCTTTA 3'
Y74S, 5' TATTTCCAGCAAGCTTGGAGAGATAAGAGACTGTCCTCCAATGTAATCCCTTTA 3'
Ampicillin repair, 5' CACCACGATGCCTGCAGCAATGGCAAC 3'

The incorporation of silent *Hin*dIII restriction sites into the mutagenic oligonucleotides facilitated rapid screening of putative mutants, and the presence of the mutations was subsequently verified by DNA sequencing across the mutated region. For heterologous expression, all subunit cDNAs, which were generous gifts from Dr. David Weiss (University of Alabama) were subcloned into the pcDNA3.1 (+/-) expression vectors (Invitrogen), which allow for high level of expression of foreign DNA under control of the cytomegalovirus promoter.

Transient Transfection and Cell Membrane Preparation

tsA201 cells (DuBridge *et al.*, 1987), derivatives of the human embryonic kidney (HEK-293) cell line, were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal bovine serum (Gibco) or Fetal Clone III (HyClone) and 100 U/ml penicillin-streptomycin solution. Transient transfection was carried out using the calcium phosphate method as described by Chen and Okayama (1987) and as modified by Davies *et al.* (1998). Cultures were grown to 70% confluency and transfected with 10 μg each of the α1, β2, and γ2 $GABA_A$ receptor cDNA constructs. The cDNA constructs were mixed in 250 mM $CaCl_2$ solution before an equal volume of N, N-bis (2-hydroxyethyl)-2aminoethanesulfonic acid (BES) buffer (pH 7.02) was slowly added. The solutions were mixed well and allowed to stand (22°C) for 15 min before dropwise addition to the cells that had been fed fresh medium. Cells were maintained in a 3% CO_2 incubator for 48h prior to harvesting. Cell membranes were prepared as described (Davies *et al.*, 1998).

Equilibrium Binding Assays

$[^3H]$Ro15-4513 (23.06 Ci/mmol, NEN DuPont) binding measurements were carried out using a Hoefer manual filtration apparatus as described (Davies *et al.*, 1998). In brief, aliquots (200 μl) of cell membranes were incubated with various concentrations (0-70 nM) of $[^3H]$Ro15-4513 at 4°C for 60 min. Non-specific binding was determined in the presence of 100 μM diazepam (0.07% (v/v) DMSO final). $[^3H]$Muscimol (20.0 Ci/mmol, NEN DuPont) binding was performed using a Biologic® Rapid Filtration System (Dupont, 1984) and carried out according to the method as previously described (Agey and Dunn, 1989). Use of this system, in which the filter-washing step was reduced to 0.5 sec, permits better resolution of the lower affinity (faster dissociating) binding sites (*see* DuPont, 1984). Non-specific binding was determined in the presence of 300 μM muscimol. Data for $[^3H]$muscimol saturation were fit to a two-site saturation model and compared to a one-site hyperbola using GraphPad Software. All K_D values reported were obtained from non-linear regression analysis using saturation data. Representative Scatchard plots are included for display only (e.g. Figs. 2-5, 2-6, 2-7, 2-8). For competition experiments, $[^3H]$muscimol (10-40 nM) was incubated with a 200 μl aliquot of cell membranes and various concentrations of unlabeled GABA (0.1 nM–1 mM), muscimol (0.1 nM-100 μM) or bicuculline methochloride (1 nM-1 mM) (see Fig 2-3 for structures) for 60-90 min at 4°C. Non-specific binding was determined in the presence of 100 μM GABA or muscimol and did not exceed 20% of the total $[^3H]$muscimol binding at the highest concentrations of ligand used. Experiments to measure the potentiation of [^{3}H]flunitrazepam binding were also conducted using the Rapid Filtration System. [^{3}H]Flunitrazepam (84.5 Ci/mmol, NEN DuPont) was incubated with cell membranes and various concentrations of unlabeled GABA (0.01–100 μM) for 90 min at 4°C.

Expression in Oocytes and Two-Electrode Voltage Clamp Analysis

Oocytes from *Xenopus laevis* were prepared as described (Goldin, 1992). GABA$_A$R subunit cRNA transcripts were prepared by linearizing $\alpha1$ and $\gamma2_L$ subunit cDNAs with *Xba*I, while the $\beta2$ subunit cDNA was linearized using *Eco*RI. cRNA transcripts were generated using T7 RNA polymerase as described by Dunn *et al.* (1999). GABA$_A$ receptor containing $\alpha1$, $\beta2$ (or mutant $\beta2$) and $\gamma2_L$ subunits were expressed by injection of 50 nl (50 ng) of cRNA (Goldin and Sumikawa, 1992) into oocytes at ratios of 1:1:1. The oocytes were maintained in Barth's solution (in mM): NaCl (88), KCl (1), $CaCl_2$ (0.5), $Ca(NO_3)_2$ (0.5), $MgSO_4$ (1), $NaHCO_3$ (2.4), 4-(2-(hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (15), pH 7.4 for 2-7 days and used for electrophysiological recordings. Oocytes under two-electrode voltage clamp at a holding potential of -60 mV were perfused continuously (at a flow rate of approximately 5 ml/min) with frog Ringer's solution (in mM): NaCl (120), HEPES (5), KCl (2), $CaCl_2$ (1.8). GABA (Sigma) or muscimol (Sigma) was dissolved in frog Ringer's and standard two-electrode voltage clamp procedures were carried out using a GeneClamp 500 amplifier (Axon Instruments Inc.). To measure the sensitivity to agonists, GABA (0.001–1 mM) or muscimol (0.0001–1 mM) was applied via the perfusion system with a 3-15 min wash out period between applications to ensure full recovery from desensitization. Agonist-activated chloride currents were recorded using pClamp 6 software (Axon Instruments Inc.). Electrodes were filled with 3M KCl and had resistances of 0.5 – 2.0 MΩ in frog Ringer's. To measure the effects of bicuculline methobromide on GABA-mediated current, bicuculline methobromide was preperfused for 3 min prior to application of GABA and bicuculline in combination.

Data Analysis

Saturation, competition, and concentration-response curves for both radioligand binding and electrophysiological experiments were analyzed by non-linear regression techniques using GraphPad Prism Software. [^{3}H]Ro15-4513 binding data were fit by the equation:

$$B = \frac{B_{\max}[L]}{K_D + [L]}$$

where B is the amount of [^{3}H]Ro15-4513 specifically bound at concentration [L], B_{max} is the maximal binding at saturating concentrations of [^{3}H]Ro15-4513 and K_D is equilibrium dissociation constant.

[^{3}H]Muscimol binding data were fit by the above equation or by the following equation:

$$B = \frac{B_{\max H}[L]}{K_{DH} + [L]} + \frac{B_{\max L}[L]}{K_{DL} + [L]}$$

where B is the amount of [^{3}H]muscimol specifically bound at concentration [L], B_{maxH} is the maximal binding at the high affinity site and B_{maxL} is the maximal binding at the low affinity site at the maximum concentrations of [^{3}H]muscimol used in these experiments. K_{DH} is equilibrium dissociation constant of the high affinity site, while K_{DL} is the dissociation constant of the low affinity site. Scatchard plots for [^{3}H]muscimol were generated by linear regression analysis (Y62F and Y62S) using GraphPad Prism Software or by the following equation (WT, Y74F, Y74S) which has been described (Rodbard and Feldman, 1975):

$$\frac{B}{F} = \frac{1}{2}\{(R1 - B)/K_{DH} + (R2 - B)/K_{DL} + \sqrt{\{[(R1 - B)/K_{DH} - (R2 - B)/K_{DL}]^2 + 4R1R2/K_{DH}K_{DL}\}}$$

where B and F represent bound and free [^{3}H]muscimol, respectively. R_1 and R_2 are the concentration of the two classes of binding sites and K_{DH} and K_{DL} are the corresponding dissociation constants.

Potentiation of specific [^{3}H]flunitrazepam binding by GABA was fitted to the equation:

$$B = B_o + \frac{(B_{\max} - B_o)10^{Xn}}{10^{Xn} + 10^{Cn}}$$

where B is the measured specific binding of [^{3}H]flunitrazepam, [L] is the logarithm of the concentration of GABA; B_{max} and B_o are the specific binding in the presence of the maximally effective concentration and in the absence of GABA. n is the Hill coefficient.

Competition curves were fit according to the following equation:

$$\theta = \left(1 + \frac{[I]}{IC_{50}}\right)^{-1}$$

where θ is the fractional amount of [^{3}H]muscimol bound in the presence of the inhibitor at a concentration [I] compared with that in the absence of inhibitor and IC_{50} is the concentration of inhibitor where $\theta = 0.5$. K_I values from competition experiments were calculated from the IC_{50} values using the Cheng-Prussof correction (1973):

$$K_I = \frac{IC_{50}}{1 + \frac{[L]}{K_D}}$$

The agonist concentration dependence of the observed current of recombinant receptors expressed in *Xenopus* oocytes were fit by the equation:

$$I = \frac{I_{\max}[L]^n}{EC_{50}{}^n + [L]^n}$$

where I is the measured amplitude of the current, [L] is the agonist concentration, EC_{50} is the agonist concentration producing 50% of the maximal response (I_{max}) and n is the Hill coefficient. In each experiment, the current (I) was normalized to the I_{max} and the normalized data were presented as % control to construct concentration-response curves.

Statistical Analysis

All data were statistically analyzed using a one-way analysis of variance (ANOVA) followed by a *post hoc* Dunnett's test to compare mutant and wild-type receptors. Bicuculline data were analyzed with a one-way repeated measures ANOVA to compare GABA-mediated chloride conductance for mutant and wild-type receptors in the absence and presence of the antagonist.

RESULTS

Equilibrium Binding Assays

The binding of [^{3}H]Ro15-4513 to wild-type and mutant receptors (Fig. 2-4) was measured to ensure that the observed effects of the $\beta 2$ subunit mutations were specific for the high affinity muscimol/GABA site and that the amino acid substitutions had not compromised the overall structure of the recombinant GABA$_A$R. In all mutant receptors, [^{3}H]Ro15-4513 recognizes a single class of high affinity benzodiazepine sites with K_D values that are not significantly different from control values (see legend to Fig. 2-4).

Cells transfected with cDNA constructs encoding the wild-type $\alpha 1$, $\beta 2$ and $\gamma 2_L$ subunits revealed a heterogeneity in equilibrium [^{3}H]muscimol binding which is consistent with the presence of two classes of independent sites for this ligand (Fig. 2-5). The K_D values for [^{3}H]muscimol in receptors containing mutations at position 74F (Fig. 2-6) and Y74S (Fig 2-7) were not significantly different from controls (Table 2-1). In each case, a two-site binding model provided a statistically better fit than a one-site model with P values for these comparisons ranging from $P<0.05$ to $P<0.005$ (data not shown). In contrast, the binding data for receptors carrying the Y62F and Y62S are adequately described by a one-site binding isotherm, with no evidence for two classes of sites. For the Y62F mutant, a single class of sites was observed with a K_D of 57.4 ± 11.1 nM (Fig. 2-8) which is significantly ($P<0.01$) increased compared to the WT high affinity control (8.9 ± 0.5 nM). The intermediate affinity (as shown by the linear Scatchard plot, Fig. 2-8) does not permit resolution of high affinity sites and any low affinity sites that may be present (see legend to Fig. 2-8). However, as described below, this mutant retained allosteric coupling between the low affinity GABA sites and the benzodiazepine site. No specific high affinity binding was measurable in the Y62S mutant but low affinity binding was retained (Table 2-1 and Fig. 2-8). This may be explained by a complete loss of high affinity sites as a consequence of the mutation. However, we cannot exclude the possibility that the sites remain present but that their affinity is reduced to an extent that they cannot be distinguished from the low affinity component. In this respect, it should be noted that technical restraints (i.e. inability to saturate the low affinity binding sites due to reduced specific binding at concentrations of muscimol >500 nM) preclude an accurate determination of the number of low affinity sites in different preparations. Furthermore, K_D values obtained for low affinity binding are subject to large error since the maximum concentration of [^{3}H]muscimol that can reasonably be used in these experiments is about 500 nM (*see* Agey and Dunn, 1989) beyond which the measure of non-specific binding exceeds that of specific binding (Wang *et al.*, 1979; Olsen *et al.*, 1981; Shank *et al.*, 1990). The B_{max} values (pmol/mg ± SEM) for the high affinity binding sites in which the WT, (1.6 ± 0.2), Y62F (1.1 ± 0.2), Y74F (0.7 ± 0.5) and Y74S (1.0 ± 0.2) β2 subunits were expressed were not significantly different. The binding density for Y62S (1.8 ± 0.9) as fit by a single binding isotherm was not significantly different.

The effects of the Y62F mutation on the high affinity binding sites were further confirmed by carrying out competition experiments with unlabelled GABA, muscimol and bicuculline methochloride. These experiments were designed to avoid significant occupancy of low affinity sites ([^{3}H]muscimol ≤ 40 nM), thereby allowing examination of the high affinity sites without complications from the second class of sites. These experiments were not possible with the Y62S mutant, which lacked measurable binding in the high nanomolar range. The Y62F mutation resulted in a 2.8 and 2.6 fold decrease in affinity for muscimol and GABA, respectively (Table 2-2). The K_I value for muscimol-induced displacement of [^{3}H]muscimol from the Y62F receptor is in excellent agreement with the directly measured $K_{D(H)}$ value (Table 2-1), confirming that the mutation significantly reduced the affinity of these binding sites. Two classes of sites for bicuculline methochloride were measured in muscimol displacement experiments using the WT and Y62F receptors, but only one class of sites was seen in the Y74F and Y74S mutants (Table 2-2 and Fig. 2-9). This apparent heterogeneity suggests a non-equivalence in bicuculline binding and that Y74 of the β subunit may play a role in this. However, this observation has not been further explored.

It has been established that agonist occupancy of their low affinity sites allosterically modulates the binding of benzodiazepine site ligands (Tallman *et al.*, 1978). The ability of GABA to potentiate [^{3}H]flunitrazepam binding therefore provides an independent measurement of the presence of these sites and of the integrity of coupling properties. Micromolar concentrations of GABA significantly potentiated 2 nM [^{3}H]flunitrazepam binding in all mutants (Table 2-10) and neither the E_{max} nor EC_{50} value for any recombinant receptor was significantly different from control values (Table 2-3). Fig. 2-10 shows the potentiation of [^{3}H]flunitrazepam binding by GABA in the wild-type receptor (Fig. 2-10A) and in the Y62F (Fig. 2-10B) and Y62S (Fig. 2-10C) mutants. The EC_{50} and E_{max} values are in excellent agreement with those obtained in $\alpha1\beta3\gamma2$ receptors in which the maximum potentiation was reported to be approximately 150% and the EC_{50} was 0.5 ± 0.1 μM (Slany *et al.*, 1995). These data indicate that the β subunit mutations do not compromise low affinity agonist binding and this provides further evidence for the presence of distinct high and low affinity binding domains in these receptors.

Two-Electrode Voltage Clamp Analysis

The functional effects of all mutations were investigated using two-electrode voltage clamp analysis of receptors expressed in *Xenopus* oocytes. Concentration-response data for GABA and muscimol are presented in Table 2-4 and Fig. 2-11. For all receptors, muscimol was, as expected, more potent than GABA in receptor activation (Amin and Weiss, 1993). The changes observed in potency paralleled the changes seen in binding affinity. Neither of the Y74 mutations affected the concentration dependence of either agonist. However, significant rightward shifts in activation were observed in both Y62 mutants, with the phenylalanine substitution producing a 2 fold shift in EC_{50} values for GABA and muscimol, and serine giving a more pronounced rightward shift (approximately 6-fold) (Fig. 2-12). The Hill slopes (n_H) are not significantly different from the wild-type, with the exception of Y74F, in which the value is significantly decreased for GABA. The significance of this change in n_H is difficult to interpret because the Hill slope is a function of both ligand binding and channel gating (Amin and Weiss, 1993). However, it is likely that the change in n_H reflects alterations in the channel gating mechanisms of the receptor since the maximum current (I_{max}) elicited in these receptors was significantly lower than all others (Table 2-4). Thus, it is possible that this amino acid substitution shifted the conformation of the receptor such that the observed cooperativity for GABA activation of the channel is lost, the implication of which may be that these putative high affinity agonist-binding sites are coupled, in some fashion, to other GABA binding domains that are essential for gating. It is equally possible that that this mutation changes the efficacy of GABA and muscimol such that they become partial agonists.

Although lack of specific high affinity binding precluded measurement of the affinity of the antagonist, bicuculline, in $\alpha1\beta2(Y62S)\gamma2L$ receptors, we were able to measure functional antagonism of GABA-gated chloride conductance by this same compound. The reported concentration for antagonism of GABA-gated currents in *Xenopus* oocytes is approximately 1 μM (Sigel *et al.*, 1990). After stabilizing the response of the cell by administration of 1 mM GABA, the response of GABA at a concentration equivalent to the EC_7 was recorded over three successive applications. Then, the response to GABA was recorded in the presence of bicuculline. Bicuculline, at this concentration, significantly depressed the maximum amplitude of the current that was not significantly different among WT (44.9 ± 6.2%), Y62F (36.2 ± 2.0%) and Y62S (41.8 ± 2.4%) (Figs. 2-13, 2-14). In each case, the I_{max} returned to control levels after an 8 min wash-out period.

DISCUSSION

The elucidation of the mechanisms underlying GABA$_A$R function is important for the understanding of inhibitory synaptic transmission in the central nervous system. The aim of the present study was to identify residues within a specific domain of the β2 subunit of the GABA$_A$R that contribute to high affinity muscimol binding and to define, in part, potential roles of this site in receptor function. Although the subunit stoichiometry of native receptors is unknown, most recent evidence indicates that the recombinant α1β2γ2 GABA$_A$R contains 2α, 2β and 1γ subunit (Chang *et al.*, 1996; Tretter *et al.*, 1997; Farrar *et al.*, 1999). One likely arrangement of these subunits within the pentamer has been suggested to be α–β–α–γ–β (Sigel and Buhr, 1997; Tretter *et al.*, 1997). This arrangement (see Fig. 2-1) provides one α–γ interface where the benzodiazepine site is thought to be located and two β–α interfaces, each of which may carry a low affinity agonist site (Sigel *et al.*, 1992; Amin and Weiss, 1993). The presence of two low affinity sites would be consistent with a Hill coefficient of approximately 2 for channel activation (*see* Macdonald and Olsen, 1994). This leaves two additional interfaces (α–β and γ–β), which could potentially form high affinity muscimol/GABA sites. According to the popular "loop model" (see Introduction), loop D contributing to these sites would be found in the N-terminal domain of the β subunit. Previous work has shown that residues within this domain of the γ and α subunits are determinants of benzodiazepine (Buhr *et al.*, 1996) and low affinity GABA (Sigel *et al.*, 1992; Smith and Olsen, 1994) binding respectively. This provided the rationale for targeting homologous residues in the β2 subunit (Y62 and Y74) to investigate their role in high affinity muscimol binding.

The major finding reported here is that Y62 of the β2 subunit is a determinant of high affinity agonist binding. Its substitution by phenylalanine reduced the affinity for both muscimol and GABA (6-fold), while its substitution by serine resulted in a dramatic reduction in affinity (>30 fold) such that no high affinity binding was measurable in this mutant. However, receptors containing the Y62S mutation were still functional, albeit with an increased EC$_{50}$ for channel activation by about 6-fold. Thus high affinity agonist binding does not appear to be obligatory for receptor activation.

In a previous study, Sigel *et al.* (1992) also mutated residue Y62 of the β2 subunit. Although these authors did not investigate receptor binding properties, they found that the Y62L mutation reduced the maximum current elicited by GABA by approximately 5-fold, leading to the conclusion that the mutation had disrupted receptor assembly. In the present study, we did not observe any reduction of the maximum current as a result of either phenylalanine or serine substitution at this position, suggesting that there were no major effects on receptor synthesis and expression.

There is no general consensus as to the number of agonist binding sites on a single $GABA_AR$ (*see* Hevers and Lüddens, 1998). There is, however, abundant evidence for the presence of high affinity sites in addition to one or more classes of sites having lower affinity (*see* Dunn *et al.*, 1994, Sieghart, 1995). Previous studies have demonstrated that there are approximately twice as many high affinity sites for muscimol as for flunitrazepam (Sigel *et al.*, 1983; Sigel and Barnard, 1984), suggesting that there are two high affinity sites per receptor. As described above, we predict that these sites are located at the α–β and γ–β interfaces, which, by their nature, are non-equivalent. While we have detected no heterogeneity in high affinity [^{3}H]muscimol binding, the bicuculline displacement experiments (Table 2-2 and Fig. 2-9) suggest that in the wild type receptor, this antagonist may discriminate between the two putative high affinity agonist sites. Although the Y62F mutation caused a significant decrease in affinity for the agonist, bicuculline binding was apparently unaltered. This result is in agreement with the previous observation that the Y62L mutation did not affect that IC_{50} values for functional antagonism of GABA-mediated chloride conductance by bicuculline (Sigel *et al.*, 1992). Conversely, neither of the Y74 mutations reported here affected agonist binding but they did alter the characteristics of [^{3}H]muscimol displacement by bicuculline. These observations suggest that although muscimol and bicuculline compete for the same binding sites, different subsets of amino acids may be involved in the recognition of the different ligands. Alternatively, Y74 mutations may have produced changes in the conformation of the receptor that indirectly affect the binding of bicuculline. Further complexity arises from the apparent preference of bicuculline for binding to the low affinity agonist sites (Enna and Snyder, 1977; Möhler and Okada, 1978; Olsen and

Snowman, 1982; Maksay, 1994). Further studies to explore this novel observation will be required to identify the specific residues with which bicuculline interacts.

The presence of multiple agonist binding sites in the $GABA_A$ receptor raises the question of their roles in receptor function. Discrepancies between the concentrations of agonists that are required to activate the receptor and agonist affinities that are measured in equilibrium binding assays are generally thought to reflect differences in receptor conformation (i.e between the activated and desensitized states). In this and many other studies (Sigel *et al.*, 1992; Amin and Weiss, 1993; Boileau *et al.*, 1999) it has been found that micromolar concentrations of GABA and muscimol are required to open the ion channel (Fig. 2-11), suggesting that the sites involved in channel activation are of intrinsically low affinity, indeed lower than can be measured in direct equilibrium binding studies. In recent functional studies, we have found that the concentrations of GABA and muscimol that induce receptor desensitization are in good agreement with the lower affinity binding component measured directly[2]. The role of the high affinity sites, however, is less clear.

The Y62 mutations disrupted high affinity agonist binding and also increased the EC_{50} values for channel activation. It is likely, therefore, that the high affinity binding sites may play a role in the efficiency of channel activation. The EC_{50} value is a macroscopic constant that depends on several microscopic processes, including ligand binding and channel gating (Colquhoun, 1998). It is, therefore, difficult to discriminate among the various contributing factors on the basis of concentration-response curves alone. This is particularly true when complications arising from multiple classes of agonist sites are introduced. One possibility is that the high affinity sites are allosterically coupled to other domains intimately involved in channel activation and that their occupancy at low concentrations of agonists increases the affinity of the latter sites to enhance the efficiency of synaptic transmission. It has been theorized that two such non-equivalent sites, in the nAChR, provide an ideal kinetic mechanism to enhance and potentially accelerate receptor activation, which may satisfy physiological requirements for rapid activation and termination of response (Jackson, 1989).

---

[2] J.G. Newell and S.M.J. Dunn, unpublished observations.

In the present study, substitution of the tyrosine residue at position 62 by serine had a more dramatic effect than the phenylalanine substitution. Although we have not made multiple amino acid substitutions at this position, the aromaticity of the residue in this position appears to be particularly important in agonist binding. As has been previously reported for agonist binding to the nicotinic acetylcholine receptor (Dougherty and Stauffer, 1990) and for benzodiazepine binding to the GABA$_A$R (Davies *et al.*, 1998), aromatic residues may be involved in a $\pi-\pi$ stacking interaction with the ligand.

Detailed analyses of structure-function relationships without knowledge of the crystal structure of the protein should be interpreted with caution (Ward *et al.*, 1990) and requires multiple approaches, including affinity labeling, to characterize a binding site (Jacques *et al.*, 1999). As with all site-directed mutagenesis studies, the major limitation of the present study is that we cannot state with any degree of certainty whether implicated residues are directly or indirectly involved in ligand binding. However, a recent report of affinity labeling by *meta*-sulfonate benzene diazonium (MSBD) revealed the presence of nucleophilic amino acid residues on the α and β subunits that are important for the binding of muscimol (Jacques *et al.*, 1999). The authors of this study identified cysteine, histidine, and tyrosine residues as being highly reactive with this ligand. Since the displacement of [^{3}H]muscimol (12 nM) by MSBD is likely to be from a high affinity site, it is interesting to consider the possibility that Y62 may be the nucleophilic residue that reacts with MSBD. Mutagenesis in combination with this affinity label will provide a powerful biochemical means to address adequately this question. Nevertheless, our mutations do appear to be specific for the high affinity agonist site and this study provides the first evidence for the structural basis of high affinity binding that has been noted for more than twenty years.

In conclusion, we have identified residue Y62 of the β2 subunit as a determinant of high affinity [^{3}H]muscimol binding in the recombinant $\alpha1\beta2\gamma2$ GABA$_A$R. Further, we have shown that the reduction in affinity of high affinity binding site(s) does not have a large effect on receptor activation (Im *et al.*, 1997). It has previously been suggested that the nicotinic acetylcholine receptor carries sites of low and high affinity and whereas the former are involved in channel activation, the latter may be important in mediating receptor desensitisation (Dunn *et al.*, 1983). By analogy to the nAChR, the high affinity site(s) of GABA$_A$R may fulfill the same role. Other investigators have likewise suggested that two molecules of GABA are required for activation and two independent molecules of neurotransmitter are required for desensitization (Cash and Subbarao, 1987). Experiments to examine the consequences of the above mutations on the desensitization of GABA$_A$R are currently in progress.

Figure 2-1

Model of a recombinant GABA$_A$R showing putative stoichiometry (Chang *et al.*, 1996; Tretter *et al.*, 1997; Farrar *et al.*, 1999) and clockwise subunit arrangement (Tretter *et al.*, 1997), in addition to the binding sites for GABA and BZD. In the model (see Smith and Olsen, 1995), one subunit carries loops A-C, while the adjacent subunit carries loop D to form a recognition site. The arrows indicate the N-terminus to C-terminus arrangement of the subunits. Note that "loop A" of the β subunit has not been implicated in GABA binding to date.

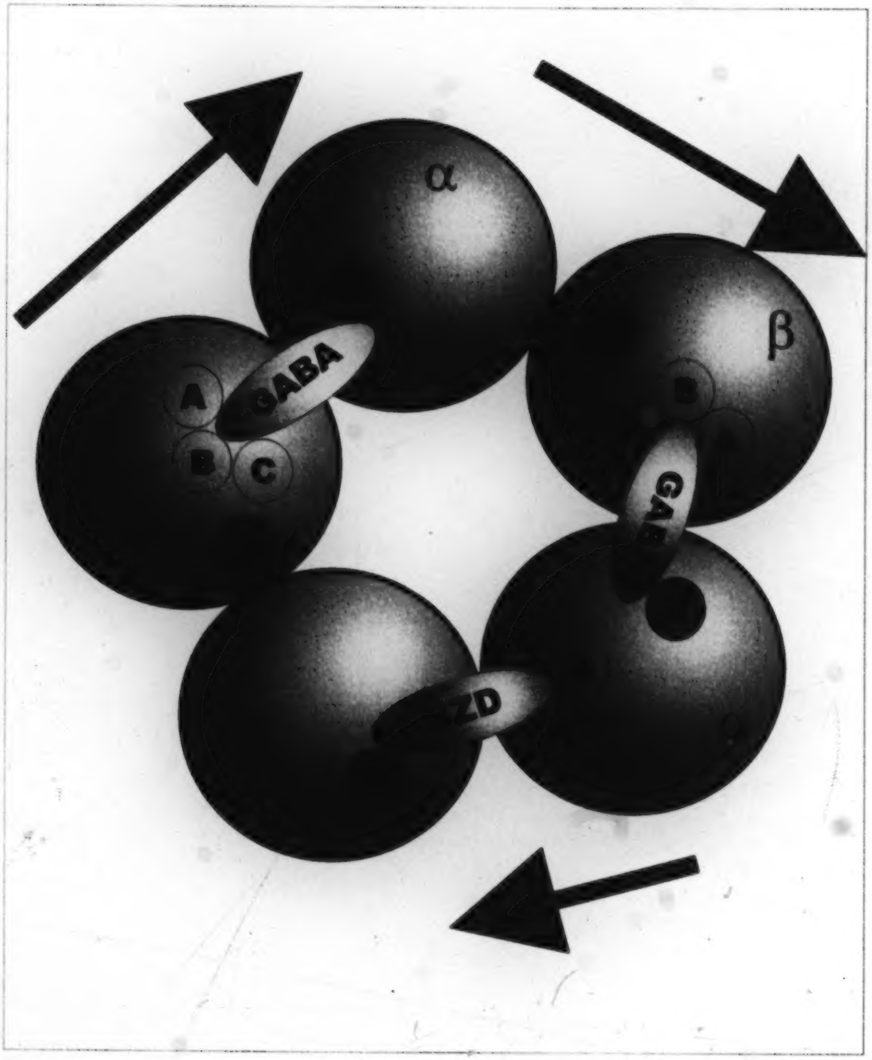

α
β
GABA
A
B
C
B
GAB
BZD
α

Figure 2-2

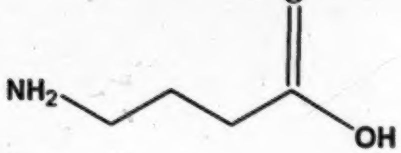
Partial amino acid sequence alignment for the rat α1-, β2-, and γ2-subunits of the GABA$_A$R, γ and δ subunits of the nAChR subunits, α subunit of the GlyR, and A subunit of the 5HT$_3$R from loop D of the amino acid sequence of these LGIC (Changeux and Edestein, 1998). The numbering shown is for the mature β2 subunit. The shaded amino acid residues have been implicated in GABA (F64: (GABA$_A$R α1), Sigel *et al.*, 1992; Smith and Olsen, 1994), benzodiazepine (F77: (GABA$_A$R), Buhr *et al.*, 1997a; Buhr *et al.*, 1997b), *d*-tubocurarine (W55/W57: (nAChR γ/δ) Pedersen and Cohen, 1990) and granisetron (W66: (5HT$_3$R A) Yan *et al.*, 1999) binding. An asterisk (*) denotes the position of the tyrosine residue of the β2 subunit that forms part of the high affinity agonist binding site.

		59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
					*													
GABA$_A$R	α1	I	D	V	**F**	F	R	Q	S	W	K	D	E	R	L	K	F	K
	β2	L	T	M	**Y**	F	Q	Q	A	W	R	D	K	R	L	S	Y	N
	γ2	I	D	I	**F**	F	A	Q	T	W	T	D	S	R	L	R	F	V
nAChR	γ	T	N	V	**W**	I	D	H	A	W	I	D	S	R	L	Q	W	N
	δ	T	N	V	**W**	I	E	M	Q	W	C	D	Y	R	L	R	W	D
5HT$_3$R	A	T	Y	I	**W**	Y	R	Q	F	W	T	D	E	F	L	Q	W	T
GlyR	α1	V	N	I	**F**	L	R	Q	Q	W	N	D	R	R	L	A	Y	N

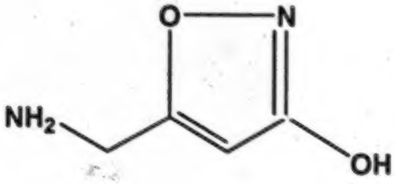
Figure 2-3

Chemical structures of the agonists, (A) GABA and (B) muscimol, and the competitive antagonist, (C) bicuculline methochloride.

A.



B.



C.

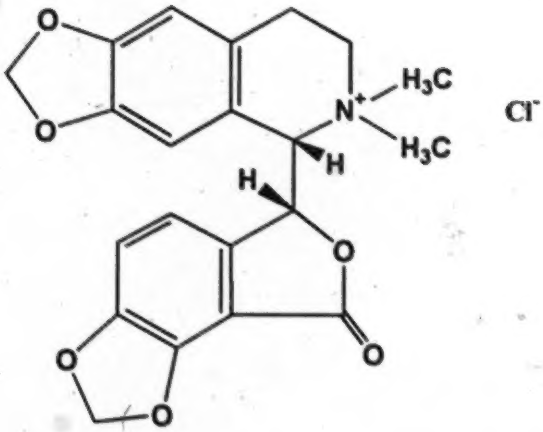

O
O
N+
H$_3$C
H$_3$C
Cl-
H
H
O
O
O
O

Figure 2-4

Saturation curves for [^{3}H]Ro15-4513 to wild-type and mutant recombinant receptors. Data represent the mean ± SEM for 4 independent experiments performed in duplicate. Data are normalized to the B_{max} value for each individual experiment. The K_D values (nM ± SEM) for WT (◆: 6.8 ± 0.8), Y62F (●: 8.1 ± 0.7), Y62S (○: 4.9 ± 0.9), Y74F (■: 5.2 ± 1.0) and Y74S (□: 3.9 ± 0.2) were not significantly different.

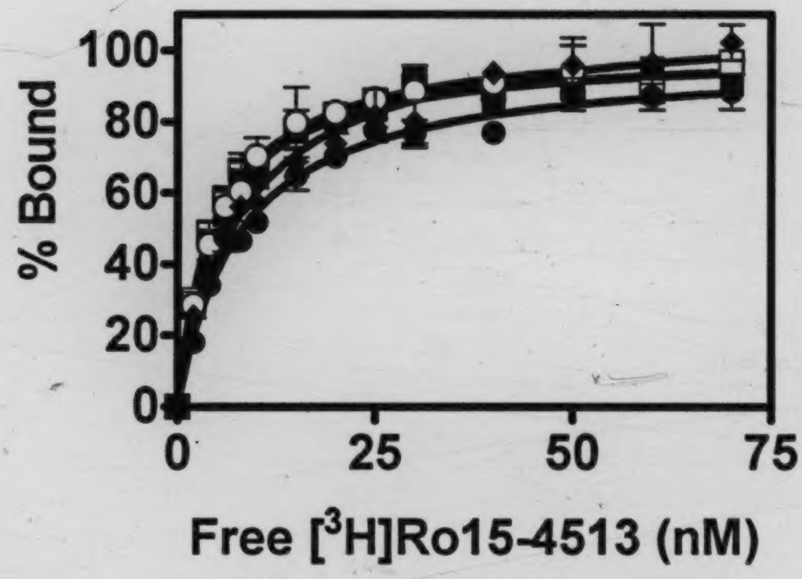
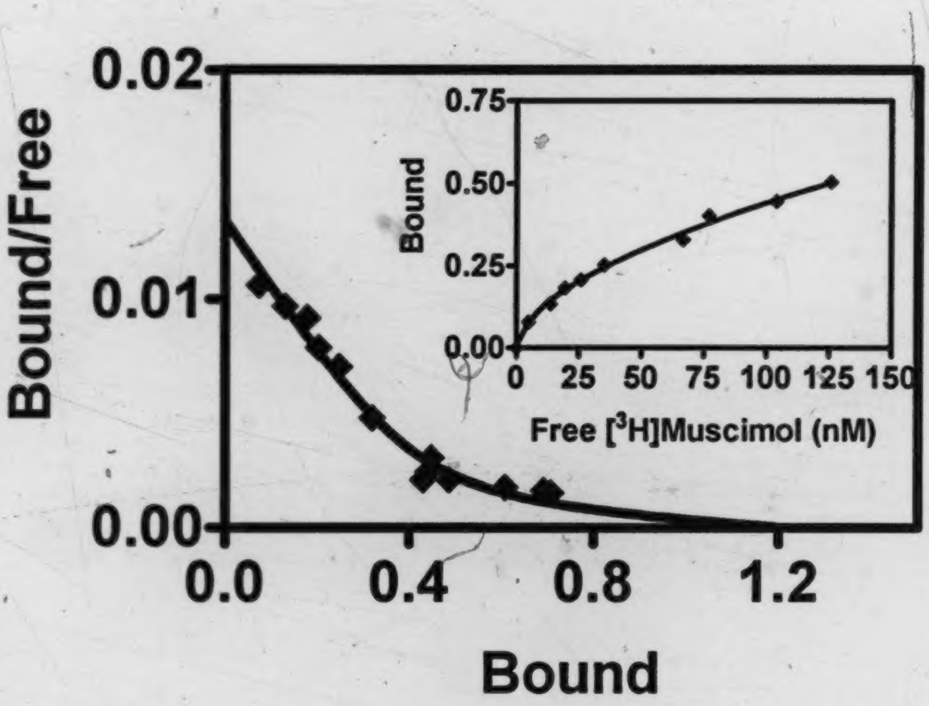

% Bound
100
80
60
40
20
0
0
25
50
75
Free [³H]Ro15-4513 (nM)

Figure 2-5

Equilibrium binding data for [^{3}H]muscimol in WT (◆) receptors. Shown are a representative Scatchard plot and a representative saturation curve (inset) (in the concentration range of 0 – 150 nM) to illustrate the shape of the curves. Data were fit by non-linear regression using GraphPad Prism Software. Comparison of two-site binding and one-site binding isotherms reveals that the two-site model better describes the data ($P<0.05$). The shallow nature of the saturation curves and the curvilinear Scatchard plots are indicative of two classes of binding sites. K_D values for muscimol saturation are summarized in Table 2-1.



Bound/Free
0.02
0.01
0.00
0.0
0.4
0.8
1.2
Bound
Bound
0.75
0.50
0.25
0.00
0
25
50
75
100
125
150
Free [3H]Muscimol (nM)

Figure 2-6

Equilibrium binding data for $[^3H]$muscimol in $\alpha1\beta2(\mathrm{Y74F})\gamma2_L$ (■) receptors. Shown are a representative Scatchard plot and a representative saturation curve (inset) (in the concentration range of 0 – 150 nM) to illustrate the shape of the curves. Data were fit by non-linear regression using GraphPad Prism Software. Comparison of two-site binding and one-site binding isotherms reveals that the two-site model better describes the data ($P<0.02$). The shallow nature of the saturation curves and the curvilinear Scatchard plots are indicative of two classes of binding sites. K_D values for muscimol saturation are summarized in Table 2-1.

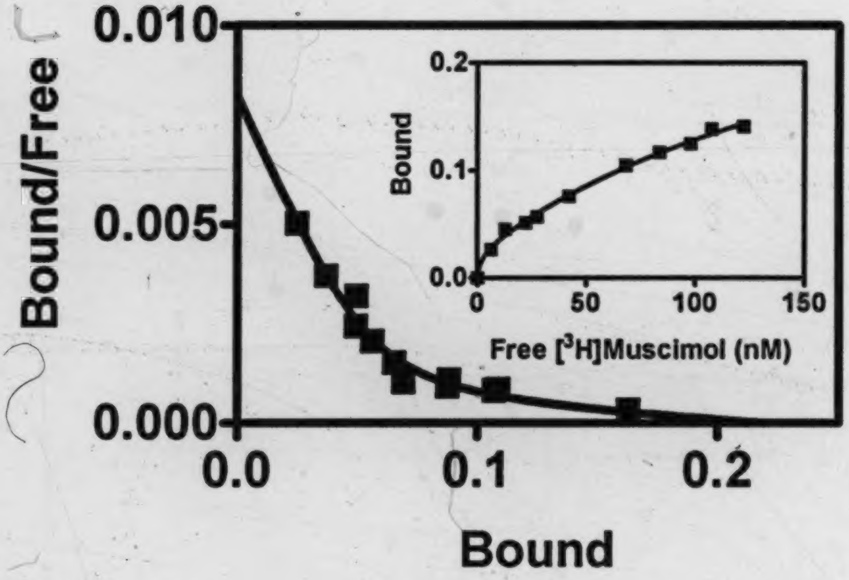
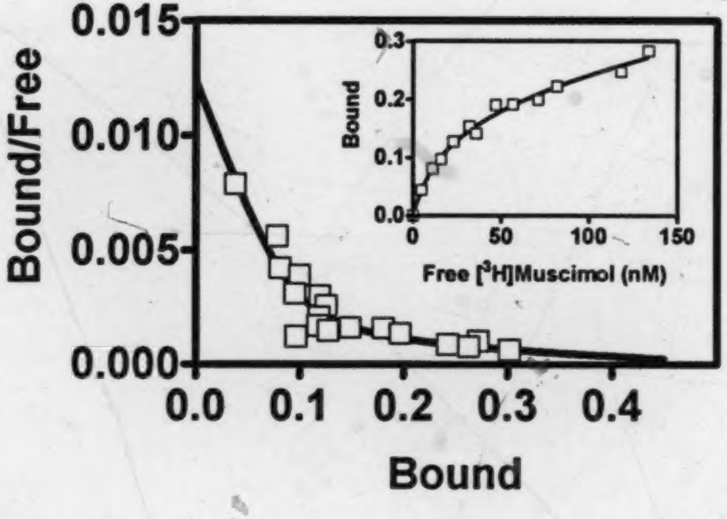

Bound/Free
0.010
0.005
0.000
0.0
0.1
0.2
Bound
Bound
0.2
0.1
0.0
0
50
100
150
Free [^{3}H]Muscimol (nM)

Figure 2-7

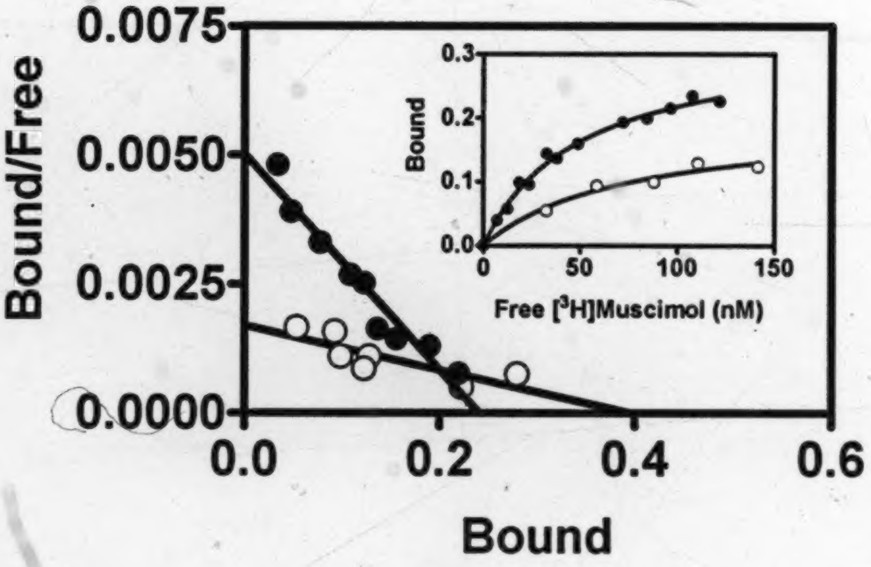
Equilibrium binding data for [^{3}H]muscimol in $\alpha1\beta2$(Y74S)$\gamma2_L$ (☐) receptors. Shown are a representative Scatchard plot and a representative saturation curve (inset) (in the concentration range of 0 – 150 nM) to illustrate the shape of the curves. Data were fit by non-linear regression using GraphPad Prism Software. Comparison of two-site binding and one-site binding isotherms reveals that the two-site model better describes the data ($P<0.005$). The shallow nature of the saturation curves and the curvilinear Scatchard plots are indicative of two classes of binding sites. K_D values for muscimol saturation are summarized in Table 2-1.



0.015
0.010
0.005
0.000
Bound/Free
0.0
0.1
0.2
0.3
0.4
Bound
0.3
0.2
0.1
0.0
Bound
0
50
100
150
Free [^{3}H]Muscimol (nM)

Figure 2-8

Equilibrium data for [^{3}H]muscimol binding to α1β2(Y62F)γ2$_L$ (●) and α1β2(Y62S)γ2L (○) mutant receptors. Shown are representative Scatchard plots and representative saturation curves (in the 0-150 nM concentration range, inset). The binding of [^{3}H]muscimol to receptors expressing these mutants was described by a one-site binding isotherm. For the purposes of illustration, a theoretical curve describing two classes of sites ($K_{D(H)}$ = 54.5 nM and $K_{D(L)}$ = 400 nM) of equivalent receptor density is also represented by the same curve for Y62F. Note that the two-site model did not better describe the data than a one-site model (K_D = 50.8 nM) within the limits of error of curve fitting by non-linear regression. The K_D values for muscimol saturation are summarized in Table 2-1.



0.0075
0.0050
0.0025
0.0000
Bound/Free
0.0
0.2
0.4
0.6
Bound
0.3
0.2
0.1
0.0
Bound
0
50
100
150
Free [^{3}H]Muscimol (nM)

Figure 2-9

Competition curves for displacement of [^{3}H]muscimol by bicuculline from recombinant receptors expressing WT (◆), Y74F (■) and Y74S (□) β2 subunits. In WT and Y62F receptors, bicuculline recognizes two apparent classes of sites with n_H of –0.59 ± 0.03 and –0.47 ± 0.06 for WT and Y62F, respectively. The fraction of sites described by the higher affinity component is 0.5 ± 0.1 for WT and 0.4 ± 0.1 for Y62F receptors. In Y74F and Y74S receptors, bicuculline displaces [^{3}H]muscimol from an apparently homogeneous population of binding sites, with n_H of –0.67 ± 0.10 and –0.98 ± 0.08, respectively. The data represent the mean ± SEM of 3-4 experiments performed in duplicate. Note that a two-site competition curve better describes the data for WT and Y62F than does a one-site model ($P<0.001$), whereas the converse is true for Y74F and Y74S ($P>0.05$). Data for all recombinant receptors for the displacement by GABA, muscimol, and bicuculline are presented in Table 2-2.

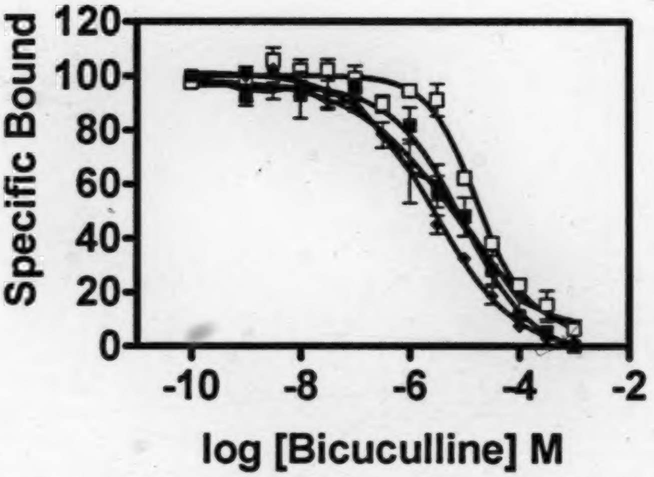

Specific Bound
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
log [Bicuculline] M

Figure 2-10

Concentration-response curve for potentiation of 2nM [^{3}H]flunitrazepam binding to $\alpha 1\beta 2\gamma 2$ receptors. A, WT (◆). B, Y62F (●). C, Y62S (○). The data represent the mean ± SEM of 4-5 experiments performed in duplicate for multiple concentrations of GABA. The maximum potentiation and the EC_{50} values of Y62F and Y62S are not significantly different from controls. The Hill slopes for WT (0.89 ± 0.22), Y62F (1.06 ± 0.70), and Y62S (1.7 ± 0.99) were not significantly different. Data for WT and mutant receptors are summarized in Table 2-3.

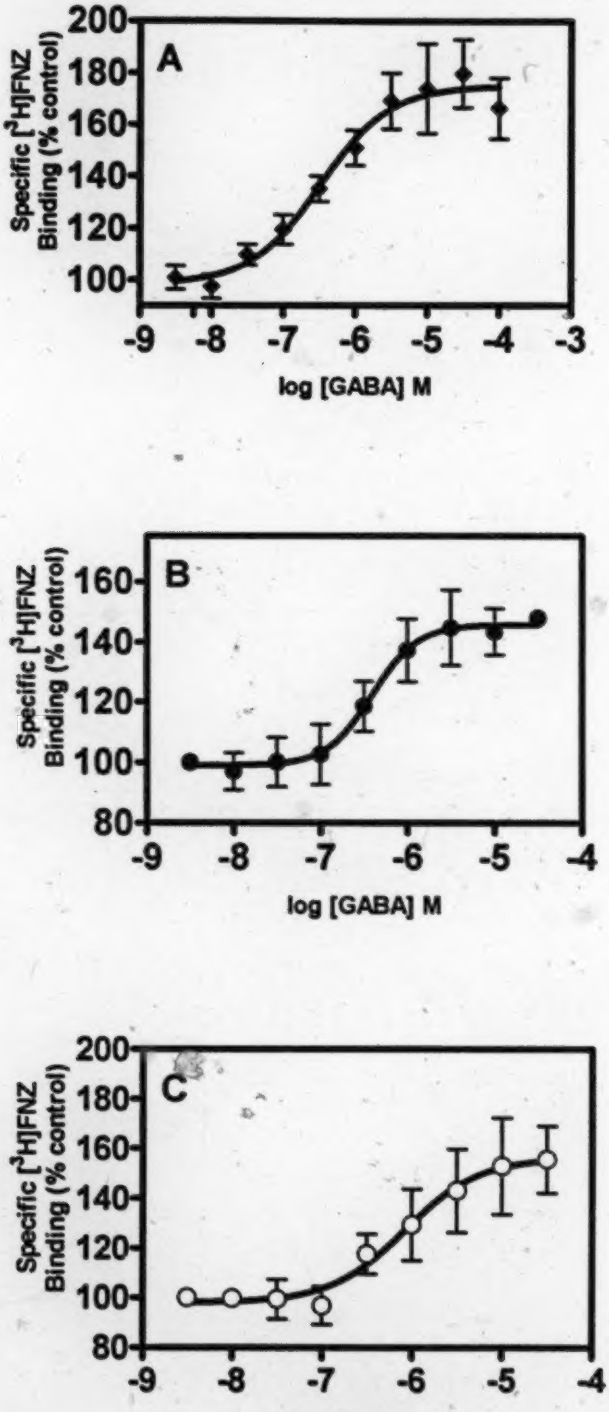
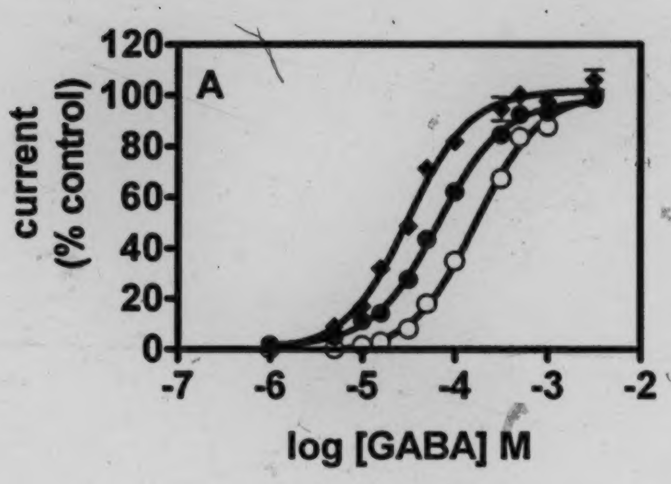

A
Specific [^{3}H]FNZ Binding (% control)
200
180
160
140
120
100
-9
-8
-7
-6
-5
-4
-3
log [GABA] M
B
Specific [^{3}H]FNZ Binding (% control)
160
140
120
100
80
-9
-8
-7
-6
-5
-4
log [GABA] M
C
Specific [^{3}H]FNZ Binding (% control)
200
180
160
140
120
100
80
-9
-8
-7
-6
-5
-4
log [GABA] M

Figure 2-11

Concentration-dependence of agonist activated Cl^- conductances for recombinant $GABA_AR$ in which WT (◆), Y62F (●), and Y62S (○) β2 subunits were expressed. Two-electrode voltage clamp analysis using *Xenopus* oocytes shows responses to GABA (A) and muscimol (B). The data represent the mean ± SEM of at least three independent experiments performed in duplicate. Data were analyzed using one-way ANOVA followed by the Dunnett's test to determine levels of significance. The shift in EC_{50} for Y62F containing mutants is approximately 2-fold for GABA and muscimol. The rightward shifts (approximately 6-fold) are more pronounced for Y62S mutants. Data for all mutants are presented in Table 2-4.


A
120
100
80
60
40
20
0
current
(% control)
-7
-6
-5
-4
-3
-2
log [GABA] M

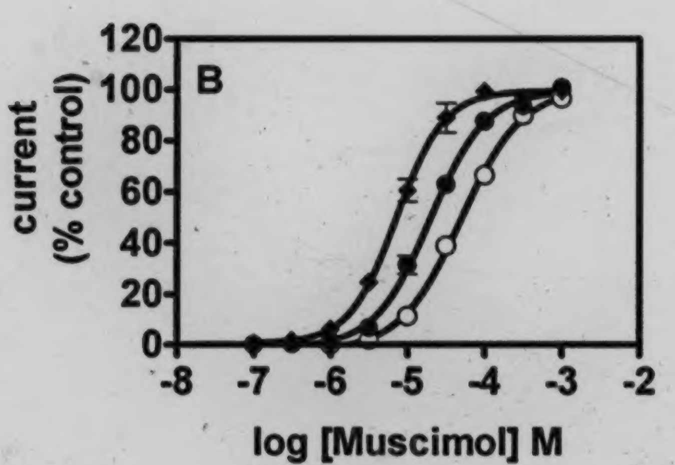

B
120
100
80
60
40
20
0
current
(% control)
-8
-7
-6
-5
-4
-3
-2
log [Muscimol] M

Figure 2-12

Representative traces showing GABA activation of recombinant GABA$_A$R expressing WT (A), Y62F (B), and Y62S (C) β2 subunits.

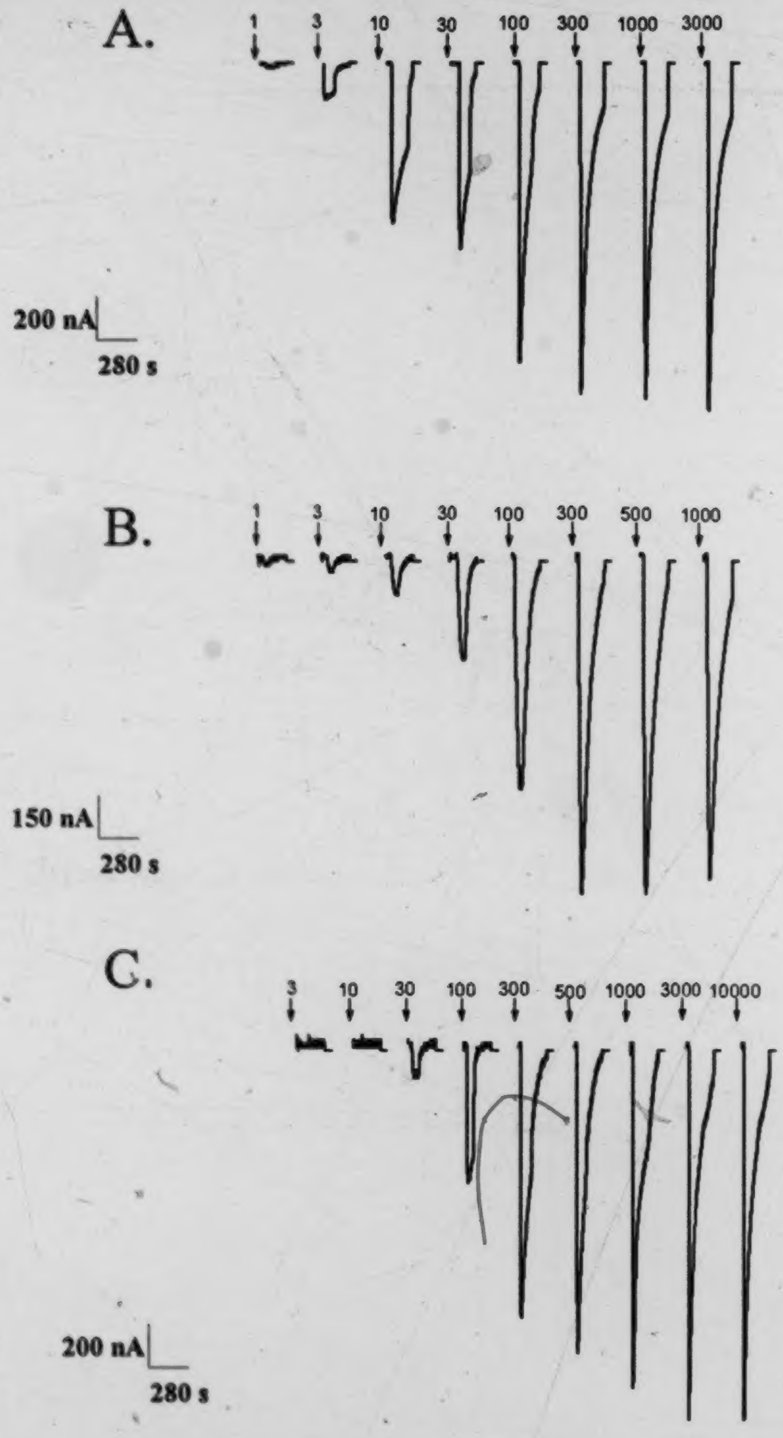

A.
1
3
10
30
100
300
1000
3000
200 nA
280 s
B.
1
3
10
30
100
300
500
1000
150 nA
280 s
C.
3
10
30
100
300
500
1000
3000
10000
200 nA
280 s

Figure 2-13

Inhibitory effects of bicuculline methobromide perfusion on GABA$_A$ mediated current in recombinant GABA$_A$R expressing WT (◆), Y62F (●), and Y62S (○) β2 subunits. Data represent the mean ± SEM of three independent experiments. The concentration of GABA used was equivalent to the EC$_7$ for receptor activation and the concentration of bicuculline was 1 μM , based on previously published reports (Sigel *et al.*, 1990; Sigel *et al.*, 1999). Bicuculline significantly depressed the current in each case ($P<0.01$) to levels that were not significantly different among WT, Y62F, and Y62S receptors (see Results).

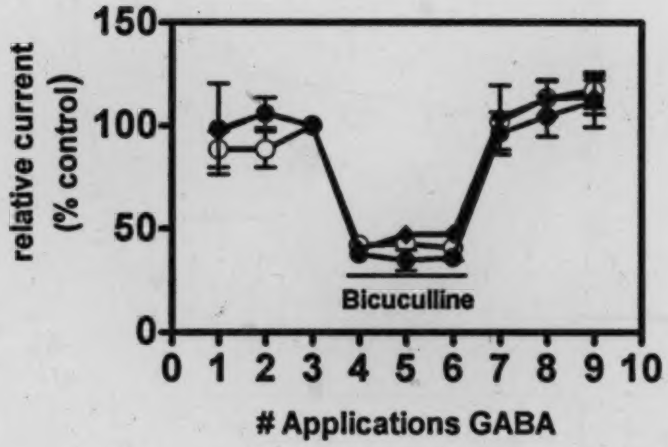

relative current
(% control)
150
100
50
0
Bicuculline
0
1
2
3
4
5
6
7
8
9
10
# Applications GABA

Figure 2-14

Representative traces showing the effects of bicuculline methobromide perfusion on GABA mediated current in recombinant $GABA_AR$ expressing WT (A), Y62F (B), and Y62S (C) β2 subunits. The concentration of GABA used was equivalent to the EC_7 for receptor activation and the concentration of bicuculline was 1 μM, based on previously published reports (Sigel *et al.*, 1990; Sigel *et al.*, 1999). Bicuculline significantly depressed the current in each case ($P<0.001$).

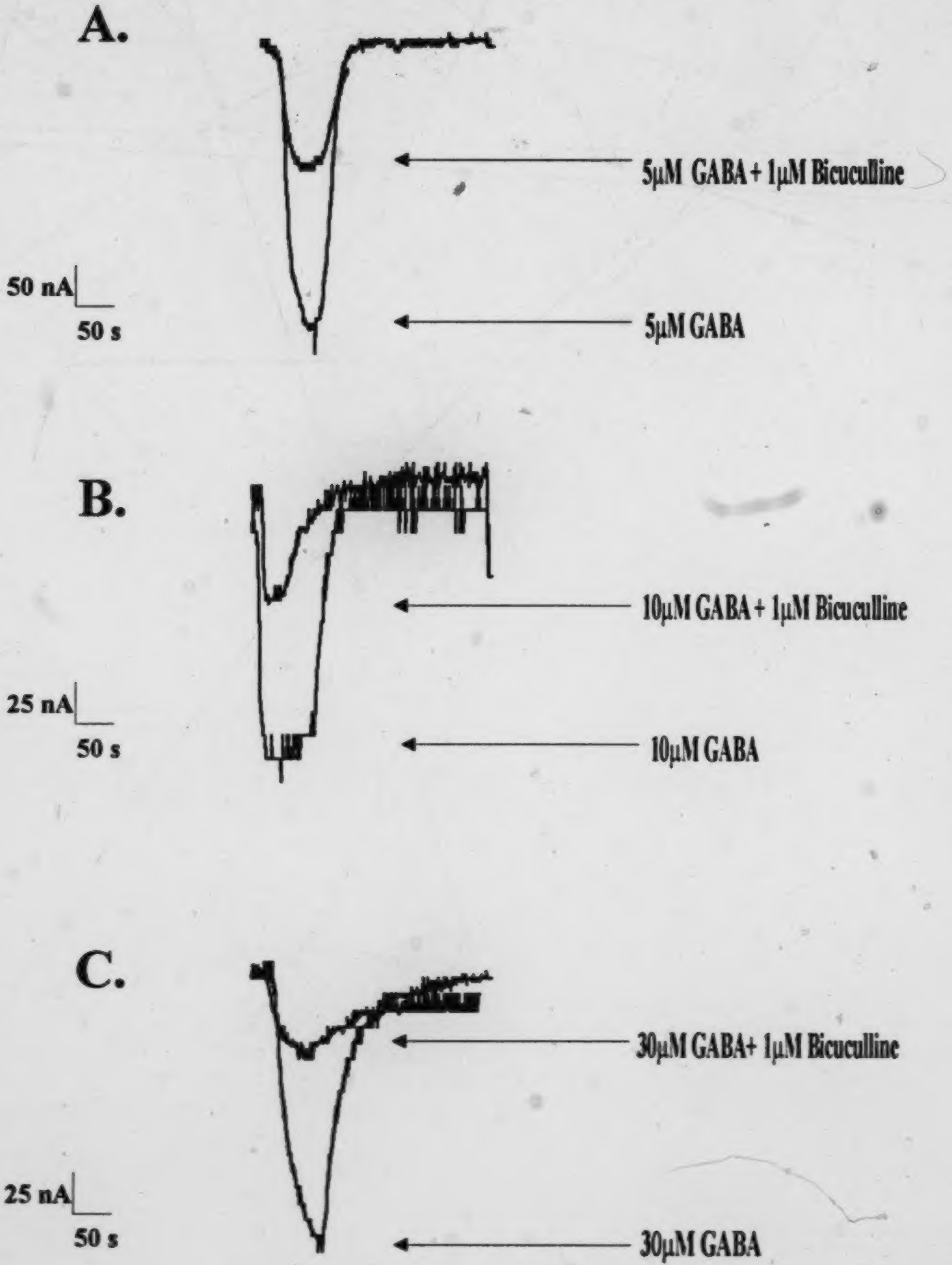

A.
5μM GABA + 1μM Bicuculline
50 nA
50 s
5μM GABA
B.
10μM GABA + 1μM Bicuculline
25 nA
50 s
10μM GABA
C.
30μM GABA+ 1μM Bicuculline
25 nA
50 s
30μM GABA

Table 2-1. The effects of amino acid substitutions in the β2 subunit of $\alpha1\beta2\gamma2_L$ recombinant $GABA_A$ receptors on high (H) and low (L) affinity $[^3H]$muscimol binding. Data represent the mean ± SEM of 2-3 independent experiments performed in duplicate using a Biologic® Rapid Filtration System. n represents the number of independent experiments. Curves were fit by non-linear regression to one-site and two-site binding models using GraphPad Prism software. K_D values were compared using a one-way ANOVA followed by a Dunnett's test to determine the levels of significance (**$P<0.01$, $^{\#}P<0.01$ when compared to $K_{D(H)}$, but not $K_{D(L)}$). No significant difference was observed for the K_D values of low affinity sites.

<table>
<tr><th>β2 subunit (n)</th><th>$K_{D(H)}$ (nM) ± SEM</th><th>$K_{D(L)}$ (nM) ± SEM</th></tr>
<tr><td>WT (3)</td><td>8.9 ± 0.5</td><td>429.1 ± 40.3</td></tr>
<tr><td>Y62F (3)</td><td colspan="2">57.6 ± 11.1**</td></tr>
<tr><td>Y62S (3)</td><td colspan="2">316.9 ± 28.4#</td></tr>
<tr><td>Y74F (3)</td><td>7.0 ± 5.2</td><td>360 ± 140</td></tr>
<tr><td>Y74S (2)</td><td>10.6 ± 5.4</td><td>1242 ± 741</td></tr>
</table>

Table 2-2. The effects of amino acid substitutions in the β2 subunit of $\alpha1\beta2\gamma2_L$ recombinant $GABA_A$ receptors on the displacement of [^{3}H]muscimol by a number of $GABA_AR$ ligands. Data represent the mean K_I ± SEM of 3-4 independent experiments performed in duplicate. Competition experiments were not carried out for Y62S as no specific high affinity binding was observed (Table 2-1 and Fig. 2-8). The number of independent experiments is shown in parentheses. log K_I values were analyzed using a one-way ANOVA followed by a Dunnett's test to determine the levels of significance (*$P<0.05$). N.D. represents no detectable bicuculline displacement. $K_{I(H)}$ and $K_{I(L)}$ represent high and low affinity bicuculline sites, respectively.

β2 subunit	Muscimol	GABA	Bicuculline Methochloride	
	K_I (nM) ± SEM	K_I (nM) ± SEM	$K_{I(H)}$ (nM) ± SEM	$K_{I(L)}$ (μM) ± SEM
WT	16.7 ± 1.2 (4)	119.2 ± 5.3 (3)	99.3 ± 54.3 (4)	4.0 ± 1.2 (4)
Y62F	47.1 ± 14.8* (4)	307 ± 47.3* (3)	41.5 ± 22.0 (3)	10.3 ± 5.1 (3)
Y74F	7.8 ± 1.3 (3)	80.8 ± 19.7 (3)	N.D.	2.9 ± 0.9 (3)
Y74S	20.2 ± 2.4 (3)	68.7 ± 16.7 (3)	N.D.	4.0 ± 0.8 (4)

Table 2-3. Potentiation of [^{3}H]flunitrazepam (2 nM) binding by GABA. Data represent the mean ± SEM of 4-5 independent experiments (n) performed in duplicate. E_{max} values and log EC_{50} values were analyzed using a one-way ANOVA followed by a Dunnett's test. No significant differences were observed for maximum potentiation or EC_{50} values.

β2 subunit (n)	E_{max} (% control)±SEM	Log EC_{50} (M)±SEM	Mean EC_{50} (nM)
WT (5)	175.0 ± 3.9	-6.48 ± 0.43	331
Y62F (4)	148.8 ± 5.2	-6.38 ± 0.24	417
Y62S (5)	156.2 ± 7.0	-6.08 ± 0.31	832
Y74F (4)	139.4 ± 7.7	-6.41 ± 0.24	389
Y74S (4)	128.8 ± 5.2	-6.60 ± 0.16	252

Table 2-4. Concentration-response data for (A) GABA and (B) muscimol activation of wild type and mutant receptors expressed in *Xenopus* oocytes. Data represent the mean EC_{50} ± SEM of 3-5 independent experiments performed in duplicate. Values for EC_{50} and Hill slope (n_H) were determined from concentration-response data using Graph Pad Prism Software. The number of independent experiments is shown in parentheses. Hill slope values and log EC_{50} values were analyzed using a one-way ANOVA followed by a Dunnett's test to determine the levels of significance (** P<0.01).

A.

β2 subunit	EC_{50} (μM) ± SEM	n_H ± SEM	$-I_{max}$ (nA) ± SEM	EC_{50} (Mutant)/ EC_{50} (WT)
WT	32.8 ± 2.5 (5)	1.33 ± 0.11	1248 ± 81.0	1.00
Y62F	65.8 ± 5.5** (3)	1.27 ± 0.09	848 ± 124	2.01
Y62S	178.4 ± 16.2** (5)	1.26 ± 0.06	707 ± 135	5.40
Y74F	26.4 ± 3.4 (4)	0.88 ± 0.07**	129 ± 11.0	0.80
Y74S	26.9 ± 2.4 (4)	1.24 ± 0.09	1516 ± 89.0	0.82

B.

β2 subunit	EC_{50} (μM) ± SEM	n_H ± SEM	$-I_{max}$ (nA) ± SEM	EC_{50} (Mutant)/ EC_{50} (WT)
WT	7.1 ± 1.0 (3)	1.32 ± 0.22	900 ± 259	1.00
Y62F	19.9 ± 3.8** (3)	1.21 ± 0.06	1753 ± 682	2.82
Y62S	48.9 ± 0.6** (3)	1.20 ± 0.40	616 ± 250	6.94
Y74F	4.7 ± 0.3 (3)	0.91 ± 0.16	127 ± 34	0.66
Y74S	5.7 ± 1.8 (3)	0.92 ± 0.02	1160 ± 218	0.80

BIBLIOGRAPHY

AGEY, M. W. AND DUNN, S.M.J. (1989). Kinetics of [^{3}H]muscimol binding to the GABA$_A$ receptor in bovine brain membranes. *Biochemistry*, **28**, 4200-4208.

AMIN, J. AND WEISS, D.S. (1993). GABA$_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature*, **366**, 565-569.

AMIN, J., BROOKS-KAYAL, A. AND WEISS, D.S. (1997). Two tyrosine residues on the α subunit are crucial for benzodiazepine binding and allosteric modulation of γ-aminobutyric acid$_A$ receptors. *Mol. Pharmacol.*, **51**, 833-841.

BOILEAU, A. J., EVERS, A.R., DAVIS, A.F. AND CZAJKOWSKI, C. (1999). Mapping the agonist binding site of the GABA$_A$ receptor: evidence for a β-strand. *J. Neurosci.*, **19**, 4847-4854.

BUHR, A., BAUR, R., MALHERBE, P. AND SIGEL, E. (1996). Point mutations of the α1β2γ2 γ-aminobutyric acid$_A$ receptor affecting the modulation of the channel by ligands of the benzodiazepine binding site. *Mol. Pharmacol.*, **49**, 1080-1084.

BUHR, A., SCHAERER, M.T., BAUR, R. AND SIGEL, E. (1997a). Residues at position 206 and 209 of the α1 subunit of γ-aminobutyric acid$_A$ receptors influence affinities for benzodiazepine binding site ligands. *Mol. Pharmacol.*, **52**, 676-682.

BUHR, A., BAUR, R. AND SIGEL, E. (1997b). Subtle changes in residue 77 of the γ subunit of the α1β2γ2 GABA$_A$ receptors drastically alter the affinity for ligands of the benzodiazepine binding site. *J. Biol. Chem.*, **272**, 11799-11804.

BUREAU, M. AND OLSEN, R.W. (1988). γ-Aminobutyric acid/benzodiazepine receptor protein carries binding sites for both ligands on both two major peptide subunits *Biochem. Biophys. Res. Comm.*, **153**, 1006-1011.

CASALOTTI, S.O., STEPHENSON, F.A. AND BARNARD, E.A. (1986). Separate subunits for agonist and benzodiazepine binding in the γ-aminobutyric acid$_A$ receptor oligomers. *J. Biol. Chem.*, **261**, 15013-15016.

CASH, D.J. AND SUBBARAO, K. (1987). Desensitization of γ-aminobutyric acid receptor from rat brain: two distinguishable receptors on the same membrane. *Biochemistry*, **26**, 7526-7270.

CHANG, Y., WANG, R., BAROT, S. AND WEISS, D.S. (1996). Stoichiometry of a recombinant GABA$_A$ receptor. *J. Neurosci.*, **16**, 5415-5424.

CHANGEUX, J.P. AND EDELSTEIN, S.J. (1998). Allosteric receptors after 30 years. *Neuron*, **21**, 959-980.

CHEN, C.A. AND OKAYAMA, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.*, **7**, 2745-2752.

CHENG, Y.-C. AND PRUSSOF, W.H. (1973). Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099-3108.

COLQUHOUN, D. (1998). Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br. J. Pharmacol.*, **125**, 924-947.

CORRINGER, P.-J., LE NOVÈRE, N. AND CHANGEUX, J.-P. (2000). Nicotinic receptors at the amino acid level. *Ann. Rev. Pharmacol. Toxicol.*, **40**, 431-458.

DAVIES, M., STEELE, J., HADINGHAM, K.L., WHITING, P.J. AND DUNN, S.M.J. (1994). Characterization of a permanently transfected cell line expressing the human $\alpha1\beta2\gamma2S$ $GABA_A$ receptor subtype. *Can. J. Physiol. Pharmacol.*, **72**, 337.

DAVIES, M., MARTIN, I.L., BATESON, A.N., HADINGHAM, K.L., WHITING, P.J. AND DUNN, S.M.J. (1996). Identification of domains in human recombinant $GABA_A$ receptors that are photoaffinity labelled by $[^3H]$flunitrazepam and $[^3H]$Ro15-4513. *Neuropharmacology*, **35**, 1199-1208.

DAVIES, M., BATESON, A.N. AND DUNN, S.M.J. (1998). Structural requirements for ligand interactions at the benzodiazepine recognition site of the $GABA_A$ receptor. *J. Neurochem.*, **70**, 2188-2194.

DENG, L., RANSOM, R.W. AND OLSEN, R.W. (1986). $[^3H]$Muscimol photolabels the g-aminobutyric acid receptor binding site on a peptide subunit distinct from that labeled with benzodiazepines. *Biochem. Biophys. Res. Comm.*, **138**, 1308-1314.

DOUGHERTY, D.A. AND STAUFFER, D.A. (1990). Acetylcholine binding by a synthetic receptor: implications for biological recognition. *Science*, **250**, 1558-1560.

DUBRIDGE, R.P., TANG, P., HSIA, H.C., LEONG, P.-M., MILLER, J.H. AND CALOS, M.P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol. Cell Biol.*, **7**, 379-387.

DUNCALFE, L.L., CARPENTER, M.R., SMILLIE, L.B., MARTIN, I.L. AND DUNN, S.M.J. (1996). The major site of photoaffinity labeling of the γ-aminobutyric acid type A receptor by $[^3H]$flunitrazepam is histidine 102 of the α subunit. *J. Biol. Chem.*, **271**, 9209-9214.

DUNN, S.M.J., CONTI-TRONCONI, B.M. AND RAFTERY, M.A. (1983). Separate sites of low and high affinity for agonists on the *Torpedo* californica acetylcholine receptor. *Biochemistry*, **22**, 2512-2518.

DUNN, S.M.J., BATESON, A.N. AND MARTIN, I.L. (1994). Molecular Neurobiology of the $GABA_A$ receptor. *Int. Rev. Neurobiol.*, **36**, 51-96.

DUPONT, Y. (1984). A rapid-filtration technique for membrane fragments or immobilized enzymes: measurements of substrate binding or ion fluxes with a few-millisecond time resolution. *Anal. Biochem.*, **142**, 504-510.

ENNA, S.J. AND SNYDER, S.H. (1977). Influences of ions, enzymes, and detergents on γ-aminobutyric acid-receptor binding in synaptic membranes of rat brain. *Mol. Pharmacol.*, **13**, 442-453.

FARRAR, S.J., WHITING, P.J., BONNERT, T.P. AND MCKERNAN, R.M. (1999). Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. *J. Biol. Chem.*, **274**, 10100-10104.

GOLDIN, A. (1992). Maintenance of *Xenopus laevis* and oocyte injection. *Methods Enzymol.*, **207**, 266-279.

GOLDIN, A. AND SUMIKAWA, K. (1992). Preparation of RNA for injection into *Xenopus* oocytes. *Methods Enzymol.*, **207**, 279-297.

HEVERS, W. AND LÜDDENS, H. (1998). The diversity of $GABA_A$ receptors. *Mol. Neurobiol.*, **18**, 35-86.

IM, W.B., PREGENZER, J.F., BINDER, J.A., DILLON, G.H. AND ALBERTS, G.L. (1995). Chloride channel expression with the tandem construct of α6-β2 $GABA_A$

receptor subunit requires a monomeric subunit of α6 or γ2. *J. Biol. Chem.*, **270**, 26063-26066.

IM, W.B., PREGENZER, J.P., BINDER, J.A. ALBERTS, G.L. AND IM, H.K. (1997). Alterations of the benzodiazepine site of rat α6β2γ2-GABA$_A$ receptor by replacement of several divergent amino-terminal regions with the α1 counterparts. *Br. J. Pharmacol.*, **120**, 559-564.

JACKSON, M.B. (1989). Perfection of a synaptic receptor: kinetics and energetics of the acetylcholine receptor. *Proc. Natl. Acad. Sci. ,U.S.A.*, **86**, 2199-2203.

JACQUES, P, PERRET, O., BOUCHET,M.-J., FOUCAUD, B., GOELDNER, M. AND BENKE, D. (1999). Irreversible site-directed labeling of the 4-aminobutyrate binding site by tritiated *meta*-sulfonate benzene diazonium. *Eur. J. Biochem.*, **264**, 189-194.

MACDONALD, R.L. AND OLSEN, R.W. (1994). GABA$_A$ receptor channels. *Ann. Rev. Neurosci.*, **17**, 569-602.

MAKSAY, G. (1994) Thermodynamics of γ-aminobutyric acid type A receptor binding differentiate agonists from antagonists. *Mol. Pharmacol.*, **46**, 386-390.

MÖHLER, H. AND OKADA, T. (1978).Properties of γ-aminobutyric acid receptor binding with (+)- [^{3}H]bicuculline methiodide in rat cerebellum. *Mol. Pharmacol.*, **14**, 256-265.

NAYEEM, N., GREEN, T.P., MARTIN, I.L. AND BARNARD, E.A. (1994). Quaternary structure of the native GABA$_A$ receptor determined by electron microscopic image analysis. *J. Neurochem.*, **62**, 815-818.

NEWELL, J.G., DAVIES, M. AND BATESON, A.N. (2000). The use of site-directed mutagenesis, transient transfection and radioligand binding: a method for the characterization of receptor-ligand interactions. *Mol. Biotech.*, **14**, 25-45.

NIELSEN, H., ENGELBRECHT, J., BURNAK, S. AND VON HEIJNE, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Prot. Eng.*, **10**, 1-6.

OLSEN, R.W., BERGMAN, M.O., VAN NESS, P.C., LUMMIS, S.C., WATKINS, A.E., NAPIAS, C. AND GREENLEE, D.V. (1981). γ-Aminobutyric acid receptor binding in mammalian brain: heterogeneity of binding sites. *Mol. Pharmacol.*, **19**, 217-227.

OLSEN, R.W. AND SNOWMAN, A.M. (1983). [^{3}H]Bicuculline methochloride binding to low-affinity γ-aminobutyric acid receptor sites. *J. Neurochem.*, **41**, 1653-1663.

OLSEN R.W., YANG, J., KING, R.G., DILBER, A., STAUBER, G. AND RANSOM, R.W. (1986). Barbiturate and benzodiazepine modulation of GABA receptor binding and function. *Life Sci.*, **39**, 1969-1976.

PEDERSEN, S.E. AND COHEN, J.B. (1990). d-Tubocurarine binding sites are located at α-γ and α-δ subunit interfaces of the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 279-289.

PREGENZER, J.F., IM, W.B., CARTER, D.B. AND THOMSEN, D.R. (1993). Comparison of interactions of [^{3}H]muscimol, *t*-butylbicyclophosphoro[^{35}S]thionate and [^{3}H]flunitrazepam with cloned γ-aminobutyric acid$_A$ receptors and of the $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ subtypes. *Mol. Pharmacol.*, **43**, 801-806.

RODBARD, D. AND FELDMAN, H.A. (1975). Theory of protein-ligand interaction. *Methods Enzymol.*, **36**, 3-16.

SIEGHART, W. (1995). Structure and pharmacology of γ-aminobutyric acid$_A$ receptor subtypes. *Pharmacol. Rev.*, **47**, 181-234.

SIGEL, E., STEPHENSON, F.A., MAMALAKI, C. AND BARNARD, E.A. (1983). A γ-aminobutyric acid/benzodiazepine receptor complex of bovine cerebral cortex: purification and partial characterization. *J. Biol. Chem.*, **258**, 6965-6971.

SIGEL, E. AND BARNARD, E.A. (1984). A γ-aminobutyric acid/benzodiazepine receptor complex form bovine cerebral cortex: improved purification with preservation of regulatory sites and their interactions. *J. Biol. Chem.*, **259**, 7219-7223.

SIGEL, E., BAUR, R., KELLENBERGER, S. AND MALHERBE, P. (1992). Point mutations affecting antagonist and agonist dependent gating of GABA$_A$ receptor channels. *EMBO J.*, **11**, 2017-2023.

SIGEL, E. AND BUHR, A. (1997). The benzodiazepine binding site of GABA$_A$ receptors. *Trends Pharmacol. Sci.*, **18**, 425-429.

SHANK, R.P., BALDY, W.J., MATTUCCI, L.C. AND VILLANI, JR. F.J. (1990). Ion and temperature effects on the binding of γ-aminobutyrate to its receptors and the high-affinity transport system. *J. Neurochem.*, **54**, 2007-2015.

SLANY, A., ZEZULA, J., FUCHS, K., SIEGHART W. (1995). Allosteric modulation of [^{3}H]flunitrazepam binding to recombinant GABA$_A$ receptors. *Eur. J. Pharmacol. (Mol. Pharmacol. Sect.)*, **291**, 99-105.

SMITH, G.B. AND OLSEN, R.W. (1994). Identification of [^{3}H]muscimol photoaffinity substrate in the bovine γ-aminobutyric acid$_A$ receptors α subunit. *J. Biol. Chem.*, **269**, 20380-20387.

SMITH, G.B. AND OLSEN, R.W. (1995). Functional domains of GABA$_A$ receptors. *Trends Pharmacol. Sci.*, **16**, 162-168.

TALLMAN, J.F., THOMAS, J.W. AND GALLAGER, D.W. (1978). GABAergic modulation of benzodiazepine binding site sensitivity. *Nature*, **264**, 383-385.

TANAY, V.A.-M.I., TANCOWNY, B.P. GLENCORSE, T.A. AND BATESON, A.N. (1997). The quantitative analysis of multiple mRNA species using oligonucleotide probes in an S1 nuclease protection assay. *Mol. Biotech.*,**7**, 217-229.

TRETTER, V., EHYA, N., FUCHS, K., AND SIEGHART, W. (1997). Stoichiometry and assembly of a recombinant GABA$_A$ receptor subtype. *J. Neurosci.*, **17**, 2728-2737.

WARD, W.H.J., TIMMS, D. AND FERSHT, A.R. (1990). Protein engineering and the study of structure-function relationships in receptors. *Trends Pharmacol. Sci.*, **11**, 280-284.

WIELAND, H.A., LÜDDENS, H. AND SEEBURG, P.H. (1992). A single histidine in GABA$_A$ receptors is essential for benzodiazepine agonist binding. *J. Biol. Chem.*, **267**, 1426-1429.

YAN, D., SCHULTE, M.K., BLOOM, K.E. AND WHITE, M.M. (1999). Structural features of the ligand-binding domain of the serotonin 5HT$_3$ receptor. *J. Biol. Chem.*, **274**, 5537-5541.

YANG, J.S.J. AND OLSEN, R.W. (1987). γ-Aminobutyric acid receptor binding in fresh mouse brain membranes at 22°C: ligand-induced changes in affinity. *Mol. Pharmacol.*, **32**, 266-277.

ZEZULA, J., SLANY, A. AND SIEGHART, W. (1996). Interaction of allosteric ligands with $GABA_A$ receptors containing one, two, or three different subunits. *Eur. J. Pharmacol.*, **301**, 207-214.

CHAPTER 3[1]

A Critical Element Of The High Affinity Agonist-Binding Domain Stabilizes the Desensitized State of GABA$_A$ Receptors

---

[1] *A version of this chapter has been submitted for publication.* NEWELL, J.G. AND DUNN, S.M.J. *J. Neurosci.*

INTRODUCTION

Desensitization is an intrinsic biophysical characteristic of ligand-gated ion channels, which facilitates neurobiological adaptation to prolonged or repeated exposure to agonist. The molecular mechanisms by which this occurs have been subject to intense investigation but remain poorly understood (Edmonds *et al.*, 1995). The general consensus is that agonist-induced conformational changes of the receptor mediate the initial, if not all, phases of the process (Ochoa *et al.*, 1989; Lohse, 1993). Although not dependent on phosphorylation (Edmonds *et al.*, 1995), desensitization can be modulated by phosphorylation of intracellular amino acid residues by protein kinase A, protein kinase C and tyrosine kinases (Huganir *et al.*, 1990). The γ-aminobutyric acid type A receptors ($GABA_ARs$) are members of a receptor gene family to which nicotinic acetylcholine receptors (nAChRs), glycine receptors (GlyRs), and the serotonin type 3 receptors ($5HT_3Rs$) belong (Barnard *et al.*, 1998). $GABA_ARs$ are large pentameric protein complexes composed of subunit isoforms from a number of classes (α1-6, β1-3, γ1-3, ρ1-3, δ, ε, π, θ). Each subunit contains a large hydrophilic amino terminus, four putative membrane spanning domains (TM1-TM4), with a large intracellular loop between TM3 and TM4 that contains consensus sequences for phosphorylation (Schofield *et al.*, 1987).

Radioligand binding studies have revealed the existence of (at least) two classes of agonist recognition sites on a single receptor molecule (Newell *et al.*, 2000a), which differ in affinity for GABA or muscimol by more than one order of magnitude (Olsen *et al.*, 1981; Yang and Olsen, 1987; Agey and Dunn, 1989). The functional consequences of agonist occupancy of these different sites remain to be clarified but it is generally assumed that the high affinity binding that is measured under equilibrium conditions reflects binding to a desensitized conformation(s) of the receptor.

Evidence that distinct high and low affinity sites may play different roles in receptor function was first suggested by biochemical studies of the prototypical *Torpedo* nAChR. This receptor is known to carry two high affinity sites, but additional low affinity sites were detected using fluorescence techniques (Dunn and Raftery, 1982). Protection of the high affinity binding sites by covalent labelling with [^{3}H]bromoacetylcholine did not prevent the receptor conformational changes associated with low affinity binding. This led to an early model in which receptor activation and desensitization are parallel receptor processes mediated by binding to distinct low and high affinity agonist sites, respectively (Raftery *et al.*, 1983). Support for the possible generality of this model came from chloride flux studies of $GABA_A$ receptors in brain homogenates, the results of which led to the suggestion that the GABA binding sites which mediate channel activation are of low affinity and distinct from higher affinity sites which may be responsible for desensitization (Cash and Subbarao, 1987a,b). However, direct tests of this model have proved difficult.

We have recently identified an amino acid residue (Y62) of the β2 subunit of the $GABA_AR$ that is important for high affinity agonist binding (Newell *et al.*, 2000a). Mutation of this residue to a phenylalanine caused a significant decrease in agonist affinity and mutation to a serine led to a loss of measurable high affinity binding sites. The mutant receptors remained functional but the EC_{50} for both GABA and muscimol-induced activation were increased by 2-fold (Y62F) and 6-fold (Y62S) compared to the wild-type receptor. These mutations thus provide the opportunity to investigate further the roles of agonist binding sites in $GABA_A$ receptor function.

In this study, we have expressed wild-type and mutant recombinant receptors in *Xenopus* oocytes and have investigated the effects of mutations of Y62 of the β2 subunit on agonist-induced receptor desensitization. We have employed a modified version of a method that has been widely used to examine the consequences of ligand binding to the desensitized state (Edelstein *et al.*, 1996). We report that mutations of Y62 to phenylalanine and serine increase the concentration-dependence of desensitization. Further, α1β2(Y62S)γ2L receptors, which display no detectable high affinity agonist binding sites (Newell *et al.*, 2000a), did not remain in the desensitized state despite the continued presence of agonist. We propose that the major role of the high affinity binding site(s) is to mediate receptor conformational changes which stabilize the desensitized state by virtue of a "locking" mechanism. This is consistent with models of receptor activation and desensitization which suggest that recovery from slow desensitization is a function of agonist dissociation from the high affinity equilibrium state of the receptor (*see* Ochoa *et al.*, 1989). In light of this evidence, we propose a model for receptor activation and desensitization that is consistent with the presence of multiple classes of agonist binding sites on a single GABA$_A$R molecule.

MATERIALS AND METHODS

Site-Directed Mutagenesis

GABA$_A$R β2 subunit mutants were engineered using the Altered Sites II® *in vitro* Mutagenesis System (Promega, Madison, WI) as previously described (Newell *et al.*, 2000a, b).

cRNA Transcript and Oocyte Preparation

Xenopus oocytes were prepared as described (Belelli *et al.*, 1996). Capped cRNA transcripts for GABA$_A$R subunits were prepared from cDNA constructs that were generous gifts from Dr. David Weiss. Oocytes were injected with α1, β2 (or β2 mutants) and γ2 subunit cRNA (1 μg/μl total RNA) in a 1:1:1 ratio in a total volume of 50 nl. Individual oocytes were maintained in 96 well plates at 14°C in modified Barth's medium (88 mM NaCl, 1 mM KCl, 0.5 mM $CaCl_2.2H_20$, 0.5 mM $Ca(NO_3)_2.4H_2O$, 2.5 mM sodium pyruvate, 1 mM $MgSO4.7H_2O$, 2.4 mM $NaHCO_3$, 15 mM HEPES, pH 7.4) that was supplemented with 100 μg/ml gentamicin.

Two-Electrode Voltage Clamp

Electrophysiological experiments were performed 2 to 7 days following oocyte injection. Standard two-electrode voltage clamp techniques were carried out using a GeneClamp 500B Amplifier (Axon Instruments Inc.) at a holding potential of –60 mV. The microelectrodes were filled with 3 M KCl and had resistances of 0.5-2.0 mΩ in frog Ringer's medium (120 mM NaCl, 1.8 mM $CaCl_2$, 2 mM KCl, and 5 mM HEPES, pH 7.4). For experimental procedures, oocytes were continuously bathed in frog Ringer's via a gravity perfusion system at a flow rate of approximately 5 ml/min. GABA (Sigma, St. Louis, MI) or muscimol (Sigma, St. Louis, MI) were dissolved in the same medium.

Pre-perfusion Protocols

The current amplitude elicited by a saturating concentration of GABA (1 mM) was stabilized prior to experimentation such that a maximum variation of 5% was observed over three successive applications. For agonist-induced desensitization experiments, oocytes were successively challenged with a 30 sec application of a concentration of GABA (1 mM) or muscimol (2 mM) that elicited a maximal response in all recombinant receptors. Pre-perfusion with lower concentrations of agonist was started after recovery of receptors from the desensitization induced by the challenge dose. The relative current amplitude used in determination of IC_{50} values for desensitization was defined by the steady state current that was achieved in the presence of the indicated agonist concentration in the pre-perfusate (see example in Fig. 3-1). The currents used to determine the stabilized desensitized state met the same criterion of stability as above before proceeding to the next concentration. Data were analyzed by iterative curve fitting using the following equation (GraphPad Prism Software):

$$I = \frac{I_{\max}[L]^n}{EC_{50}{}^n + [L]^n}$$

where I is the measured amplitude of the current, $[L]$ is the agonist concentration, EC_{50} is the agonist concentration producing 50% of the maximal response (I_{max}) and n is the Hill coefficient. In each experiment, the current (I) was normalized to the I_{max} and the normalized data were presented as % control to construct concentration-response curves

For experiments to measure the effects of frequency of challenge and duration of pre-perfusion on current amplitude, desensitizing GABA concentrations equivalent to their IC_{50} values were used in the pre-perfusion. Control currents to GABA were measured in the absence (control) and presence of GABA in the pre-perfusate to demonstrate that desensitization was a function of agonist in the bathing medium and not of current rundown. Unlike the methods described above, pre-perfusion was started immediately during the recovery phase from the previous agonist challenge. The maximum depression of current amplitude that was attained using either pre-perfusion protocol was not different. Experiments demonstrating recovery from agonist application were carried out using a challenge concentration of GABA that was equivalent to its EC_{50} for receptor activation. Recovery from the desensitized state was measured as a function of time (0-80 min).

Statisitical Analyses

Data were analyzed by a one-way analysis of variance (ANOVA) followed by either a *post-hoc* Dunnett's test or Newman-Keuls to determine levels of significance. The data for GABA-mediated chloride conductance (Figs. 3-2A, 3-3A,B) were analyzed by a one-way repeated measures ANOVA to compare the current before and after pre-perfusion of a concentration of GABA equivalent to the IC_{50} value for agonist-induced desensitization.

RESULTS

Agonist-Induced Desensitization

Representative two-electrode voltage clamp recordings from $\alpha 1\beta 2\gamma 2_L$ recombinant receptors expressed in *Xenopus* oocytes show that repeated application of a maximally effective concentration of GABA (1 mM) at 12-min intervals (see below) elicits a rapidly desensitizing current (Fig. 3-1). We have previously reported that the EC_{50} for GABA-activated currents in this receptor subtype is 32.8 ± 2.5 μM with a Hill coefficient of 1.33 ± 0.11 (Newell *et al.*, 2000a). Fig. 3-1A shows the effects of bath perfusion with 10 μM GABA i.e., a concentration which itself evokes a small current response (~15% of maximum). After perfusion for 12 min, the amplitude of the current induced by the challenge dose was reduced. In the continued presence of the lower GABA concentration, the currents declined to a steady state level but recovered to the original level when GABA was removed from the perfusion medium. We interpret the observed reduction in current as receptor desensitization induced by GABA in the perfusion medium (see below).

Fig. 3-1B shows the effects of bath perfusion of a lower concentration of GABA (1 μM) which by itself induces only a minimal chloride conductance (<2% of maximum). In contrast to the results in Fig. 3-1A, there was no diminution of the response to the first GABA challenge; rather a small, but reproducible, increase in the current amplitude was observed under these circumstances. However, upon repeated challenges, the current again declined to a steady state level and this was fully reversible upon removal of GABA from the bath perfusion. These data suggest that, at low agonist concentrations, the extent of desensitization depends on the number of receptor activations (*see* also Bartrup and Newberry, 1996).

Optimization of the Experimental Conditions to Investigate Desensitization

The experimental protocol used here to study $GABA_A$ receptor desensitization was adapted from that of Bartrup and Newberry (1996) who used whole cell patch clamp techniques to study agonist-induced desensitization of the $5HT_3$ receptor in NG108-15 cells. Since $GABA_A$ receptor desensitization has not previously been quantified using the *Xenopus* oocyte system, we have optimized the experimental procedures to ensure that any desensitization observed was due to the presence of agonist in the perfusate and was not a function of either current rundown or the frequency of agonist challenge. Fig. 3-2A shows the effects of changing the time interval between challenges. As in Fig. 3-1, currents to a maximally effective GABA concentration (1 mM) were recorded at regular time intervals but, in these experiments, this interval was varied between 3 and 12 min as indicated. After the third challenge, the oocyte was perfused with 3 μM GABA, a concentration that approximates its IC_{50} for desensitization (see below). Responses to subsequent regular challenges were then recorded prior to removal of the GABA from the perfusion medium. Using a 9-min or 12-min interval, the control responses were reproducible in amplitude and there was no difference in the magnitude of the depression observed during agonist perfusion or in the rate or extent of recovery upon washout. A 3-min interval is clearly not suitable for measuring desensitization (Fig. 3-2A), since the receptor is unable to recover from the desensitization induced by the challenge itself. Using a 6-min interval, there were obvious frequency-dependent effects on washout i.e., the current did not immediately return to control levels. Similar control experiments have been carried out using muscimol (data not shown). In all cases, a 12-min interval between challenges gave rise to reproducible effects in which the apparent desensitization depended only on the presence of agonist in the perfusion medium and on the number (as noted above) but not the frequency of channel activations.

The effects of the time of pre-perfusion were also investigated. Fig.3-2B shows representative experiments using wild-type receptors. In these experiments, responses to 1 mM GABA were first stabilized and, after a 12-min washout period, the oocytes were perfused with a 3μM desensitizing concentration for different times prior to challenge. Increasing the pre-perfusion time from 1 min to 12 min showed that the extent of current depression was dependent upon perfusion time but there were no significant differences between 9-min and 12-min intervals between agonist application, suggesting that desensitization reaches a steady state within about 9 min. This also illustrates that unlike rapid application techniques (Jones *et al.*, 1995), gravity perfusion requires longer periods of equilibration to achieve a stabilized desensitized state, which is in good agreement with theoretical considerations of Edelstein *et al.* (1996).

Desensitization of GABA_A Receptors Carrying Mutations Of Y62 Of The β2 Subunit.

In receptors carrying the β2-subunit Y62F mutation, the desensitization characteristics measured in control experiments (Fig. 3-3A) were very similar to those of wild-type. However, notable differences were observed using the Y62S mutant receptor (Fig. 3-3B). For this receptor, no frequency-dependent effects were apparent using intervals between challenges of 3 to 12min, implying that this receptor recovers from desensitization more rapidly than wild-type.

Effect Of Agonist Concentration On Receptor Desensitization

Experiments similar to those illustrated in Fig. 3-1 were carried out to investigate the effects of agonist concentration on the steady state level of desensitization. Fig. 3-4A shows the concentration dependence of both GABA- and muscimol-induced desensitization of the wild-type receptor. The apparent IC_{50} values were 3.3 and 0.7 μM, respectively (see Table 3-1). In direct binding studies using [^{3}H]muscimol, we have reported that the WT receptor expressed in tsA201 cells has (at least) two classes of binding sites with affinities of 8.9 nM and 0.43 μM respectively (Newell *et al.*, 2000a). Although the latter value is subject to large error due to rapid ligand dissociation during binding assays (*see* Agey and Dunn, 1989), this apparent K_D correlates well with the IC_{50} value for muscimol-induced desensitization. This suggests that it is the binding of muscimol to its lower affinity sites measured in radioligand binding studies that may mediate the desensitization process.

The Y62F (Fig. 3-4B) and Y62S (Fig. 3-4C) mutations altered the concentration dependence for both GABA and muscimol-induced desensitization, the effects being greater in the case of the Y62S substitution (Table 3-1). In both cases, muscimol was more potent than GABA in inducing desensitization, and is also more potent in activation of these mutant receptors (Newell *et al.*, 2000a).

Receptors Carrying The β2 Subunit Y62S Mutation Do Not Maintain The Desensitized State.

During the course of the above experiments, we observed a curious property of the Y62S mutant receptor. Although this receptor still activates and desensitizes, it recovers from desensitization even in the continued presence of the desensitizing concentration of agonist. The representative traces in Fig. 3-5 illustrate this phenomenon that was reproducible in 4 independent experiments. In these experiments, the wild-type (Fig. 3-5A) or Y62S (Fig. 3-5B) receptors were challenged successively with concentrations of GABA equivalent to their EC_{50} values for activation. When the wild-type receptor was perfused with 3 μM GABA the currents, as expected from the previous results, declined to a steady state level and returned to control values upon washout. In contrast, the Y62S mutant receptor showed desensitization during the pre-perfusion but, rather than reaching a steady state residual current, the currents began to grow again even in the continued presence of 3 μM GABA in the pre-perfusate. Thus this receptor, which I have shown previously to lack measurable high affinity binding sites (Newell *et al.*, 2000a), appears unable to maintain the desensitized state. This phenomenon is concentration dependent and although it is obvious at low agonist concentrations e.g. 3 μM as in Fig. 3-5B, it is not obvious at higher concentrations e.g. 25 μM as in Fig. 3-3B.

DISCUSSION

The identification of molecular domains that contribute to receptor activation and desensitization is a primary goal in structural studies of $GABA_AR$. The number of agonist binding sites on a single $GABA_AR$ has not been unequivocally defined, owing in large part to complexities arising from the variety of methodologies and receptor preparations that have been used in studies of this nature. Radioligand binding studies of single $GABA_AR$ subtypes suggest that there are at least two classes of binding sites of high (K_D = 10-30 nM) and low nanomolar (K_D = 0.1-1.0 μM) affinity for agonists (Agey and Dunn, 1989). Although some investigators propose that these differences in affinity represent multiple interconvertible receptor states, recent evidence has clearly shown that both exist under equilibrium conditions (Davies *et al.*, 1994; Newell *et al.* 2000). In addition to these sites, many investigators have speculated (*see* Olsen *et al..*, 1981) that there may be additional "ultralow" affinity sites ($K_D > 10$ μM) to explain the micromolar concentrations of GABA that are required to activate the $GABA_AR$ ion channel. Sites of such intrinsically low affinity are almost impossible to resolve in equilibrium binding assays (Shank *et al.*, 1990) and thus their existence remains in question. Irrespective of the true number of agonist binding sites carried by each receptor, it is clear that the functional roles of the multiple classes of sites have not been adequately addressed. We now report that low affinity binding sites (measured in direct binding studies) are likely to mediate receptor desensitization, while the high affinity binding sites stabilize the desensitized state by "locking" the receptor conformation (see Fig. 3-6). The work presented here reinforces the idea that agonist sites of different affinities have unique roles in receptor function.

Several models of receptor activation and desensitization have been proposed to account for conformational transitions of allosteric proteins. Adaptations of the Monod-Wyman-Changeux model (Monod *et al.*, 1965) suggest that within a population of receptors, there exist two distinct receptor conformations in which there is an equilibrium between a low affinity "resting" state and a high affinity "desensitized" state. This model also predicts that ligand binding to the resting state promotes desensitization and the model has been extended to include both fast (i.e. sub-second) and slow (i.e. min) phases of desensitization. Fast desensitization of receptors from the active state is believed to produce a state of intermediate affinity through which receptors may cycle before achieving a slowly desensitizing state of high affinity (Monod *et al.*, 1965). However, contrary this widely accepted model, we would suggest that binding to high and low affinity sites occurs in an independent fashion, in accordance with the model of Raftery *et al.* (1983), since both classes of sites exist at equilibrium (Newell *et al.*, 2000a).

There is much evidence to suggest that desensitization of the nAChR occurs via agonist-induced transitions mediated by binding to a neurotransmitter binding site (Kuhlmann *et al.*, 1991). In the present study, there is a reasonable correlation between the concentration dependence of desensitization and the K_D for the lower affinity binding sites measured in wild-type receptors (Newell *et al.* 2000a). From this observation, it may be inferred that low affinity sites mediate desensitization. The association of desensitization phenomena with low affinity sites contradicts the speculation of many investigators that it is the high affinity agonist binding sites that mediate desensitization (Ebert *et al.*, 1996).

Classical two-state models of receptor activation suggest that desensitization can occur without channel activation; indeed this is one of the basic tenets of the Monod-Wyman-Changeux (1965) model. In the present study, bath perfusion of agonist reproducibly inhibited GABA mediated chloride conductance at recombinant $GABA_ARs$ in a concentration-dependent manner. Concentrations of GABA (e.g. 1 μM) that are likely to saturate the high affinity binding sites (K_D = 119 nM in tsA201 cells) but not low affinity sites (apparent K_D = 3.3 μM) reduced current amplitude when (1) receptor activation occurred during agonist perfusion (Fig. 3-1B) and/or (2) agonist perfusion took place during the recovery phase from agonist challenge (Fig. 3-2A). These findings may suggest that low agonist concentrations bring about receptor desensitization only when the receptors pass through the open channel state. However, the correlation between activating and desensitizing concentrations may be fortuitous and this suggestion remains speculative since the majority of kinetic models indicate that receptors can proceed to the desensitized state without being activated (Jones *et al.*, 1998).

In the case of wild-type and mutant receptors, desensitization occurred at concentrations that were approximately 8-10 fold lower than those required for channel activation (*see* Newell *et al.*, 2000). The physiological significance of agonist-induced desensitization by low neurotransmitter concentrations may be related to neurotransmitter spillover from the synaptic cleft. Ambient levels of this neurotransmitter within the extracellular space are reported to range from 0.8 μM (Lerma *et al.*, 1986) to 10 μM (Destexhe and Sejnowski, 1995). In the case of non-synaptic cross-talk, where diffusion of neurotransmitter from one synapse can influence the activity at a neighbouring synapse, there is an absolute requirement for a high affinity receptor (Huang, 1998) or at least a binding site of sufficiently high affinity on a receptor molecule to facilitate extrasynaptic transmission.

The rate of recovery of $GABA_AR$ from desensitization is presumed to be almost entirely dependent on the dissociation of GABA from receptor molecules (Clements, 1996; Jones *et al.*, 1998). Receptors expressing mutations of Y62S of the high affinity binding site recovered to control levels as a function of time during continuous agonist perfusion. Dissociation from the high affinity site would represent the final ligand unbinding step (given its high nanomolar affinity relative to low affinity sites). It would therefore be predicted that a reduction in its affinity would accelerate ligand dissociation and potentially disrupt the stabilized desensitized state. Mutations of Y62 of the β2 subunit were previously reported to have differential effects on the ligand binding affinities of GABA and muscimol such that substitution by serine abolished high affinity muscimol binding (Newell *et al.*, 2000). Consequently, this loss of high affinity agonist binding facilitated the slow recovery from the final desensitized state (Raftery *et al.*, 1983). Additional evidence from experiments examining the frequency dependence of channel activation show that α1β2(Y62S)γ2 receptors, unlike all others, recovered within 3 min of ion-channel activation. Together, these data suggest that high affinity agonist binding may serve a very novel role in receptor function i.e. locking the conformation of the receptor so as to stabilize the desensitized state.

In conclusion, we have characterized the functional consequences of mutations to the high affinity agonist binding sites of recombinant $GABA_AR$ expressed in *Xenopus* oocytes. In agreement with studies of the neuronal nAChR (Corringer *et al.*, 1998), we have observed that mutations to high affinity binding domains have profound impact on the concentration of agonist required to induce desensitization. Stepwise reduction in affinity for [^{3}H]muscimol by phenylalanine and serine substitutions produced a concomitant increase in the concentration dependence of agonist-induced desensitization. More importantly, absence of measurable high affinity binding facilitates rapid recovery from desensitization, which suggests that the high affinity binding site plays a major role in conferring stability to the desensitized state. Although it is not possible to state with certainty the mechanisms by which this phenomenon occurs, a potential mechanism is likely to be via alterations to the conformational machinery of the receptor. Confirmation of this novel result will undoubtedly require crystallization of native $GABA_AR$ and the use of high resolution biophysical techniques to measure conformational changes of the receptor. Nevertheless, we have illustrated the importance of high affinity agonist binding domains in $GABA_AR$ function as it provides, for the first time, a structural basis that recovery of $GABA_AR$ from desensitization is mediated by a high affinity GABA binding site.

Figure 3-1

Representative recordings from application of GABA to recombinant α1β2γ2 $GABA_A$ receptors expressed in *Xenopus* oocytes. The oocyte was challenged at regular time intervals (12-min) using a concentration of agonist that elicited a maximum response (1 mM). Perfusion of low concentrations of GABA in the bath results in a decrease in the maximum amplitude of the current mediated by challenge applications. Equilibrium is achieved on successive concentrations. The responses to perfusion of 10 μM (A) GABA decreases the I_{max} on the first challenge application, which suggests that desensitization occurs in the presence of agonist concentrations that can open the channel. Perfusion of 1 μM (B) indicates that desensitization does not occur until the channel has been opened by GABA.

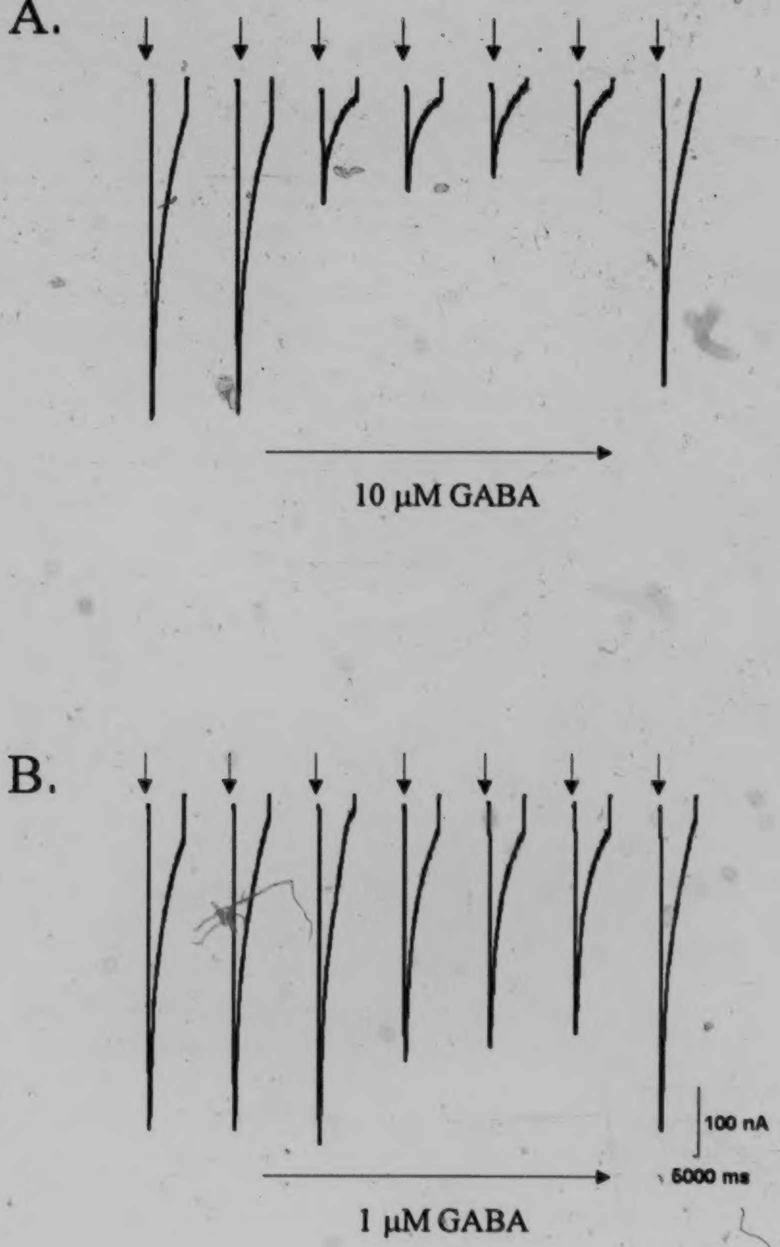
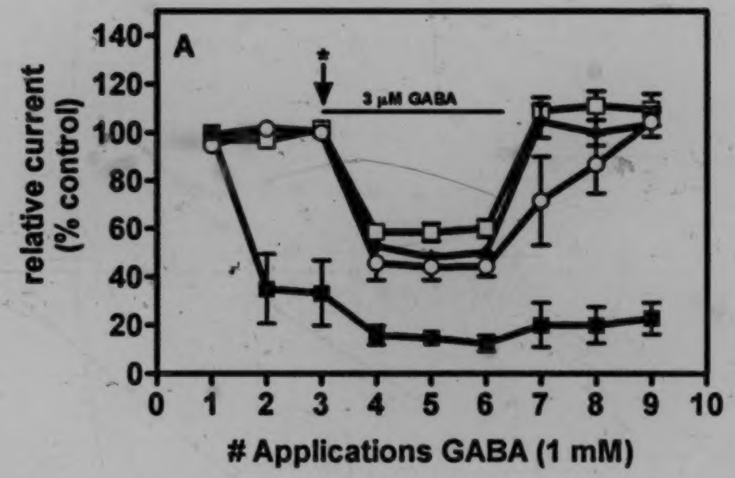

A.
10 μM GABA
B.
100 nA
5000 ms
1 μM GABA

Figure 3-2

(A) Inhibitory effects of GABA perfusion on GABA (1mM) mediated Cl^- conductances in recombinant $GABA_AR$ containing WT β2 subunits. Data represent the mean ± SEM of 4 independent experiments. Currents are normalized to the third GABA challenge (before perfusion), with the exception of the 3-min time interval in which recovery to the initial control level (i.e. application 1) did not occur. Following the third application (*) of GABA, the agonist was pre-perfused at a concentrations of 3 μM to examine the effects of time [3-min (■), 6-min (○), 9-min (◆), and 12-min(□)] on functional recovery of the response from the challenge concentration. Inhibition of GABA evoked current is significantly depressed during perfusion of agonist (see Results). These data show that functional recovery is affected differentially by the frequency of agonist application. There is no significant difference in the depression of normalized I_{max} at WT (9-min and 12-min) for the times indicated in parentheses. Frequency dependent effects are observed at 3-min and 6-min time interval in WT receptors. (B) Effect of time of agonist perfusion on GABA-gated current in α1β2γ2 receptors. In each case, GABA was pre-perfused for the time indicated and allowed to recover for 12-min before subsequent challenge. Increased pre-perfusion time significantly depressed GABA-gated current from control values, but there was no significant difference between the 9-min pre-perfusion time and the 12-min pre-perfusion time. Data represent the mean ± SEM of (n) independent experiments.


A
*
3 µM GABA
relative current
(% control)
0
20
40
60
80
100
120
140
0
1
2
3
4
5
6
7
8
9
10
# Applications GABA (1 mM)

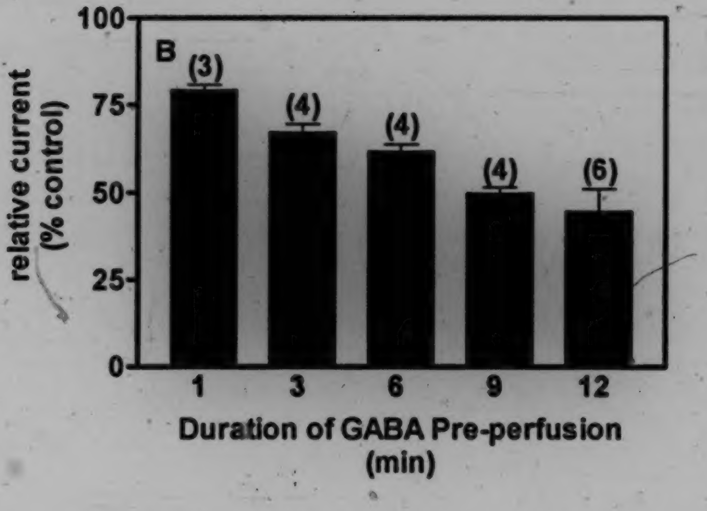
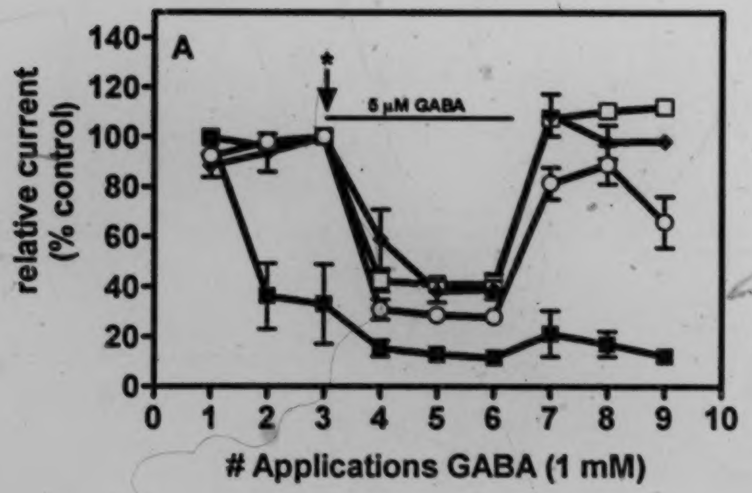

B
(3)
(4)
(4)
(4)
(6)
relative current
(% control)
0
25
50
75
100
1
3
6
9
12
Duration of GABA Pre-perfusion
(min)

Figure 3-3

Inhibitory effects of GABA perfusion on GABA-mediated (1 mM) Cl^- conductances in recombinant $GABA_AR$ containing Y62F (A) and Y62S (B) β2 subunits. Data represent the mean ± SEM of 3 (Y62F) – 4 (Y62S) independent experiments. Currents are normalized to the third GABA challenge (before perfusion), with the exception of Y62F for the 3-min time interval in which recovery to the initial control level (i.e. application 1) did not occur. Following the third application (*) of GABA, the agonist was pre-perfused at concentrations of 5 μM (Y62F) and 25 μM (Y62S) to examine the effects of time [3-min (■), 6-min (○), 9-min (◆), and 12-min(□)] on functional recovery of the response from the challenge concentration. Inhibition of GABA-evoked current is significantly depressed during perfusion of agonist (see Results). These data show that functional recovery is affected differentially by the frequency of agonist application. There is no significant difference in the depression of normalized I_{max} at Y62F (6-min, 9-min and 12-min) and Y62S (3-min, 6-min, 9-min, and 12-min) for the times indicated in parentheses. Frequency dependent effects are observed at 3-min and 6-min time interval in Y62F containing receptors.


A
*
5 μM GABA
relative current (% control)
# Applications GABA (1 mM)
0
20
40
60
80
100
120
140
0 1 2 3 4 5 6 7 8 9 10

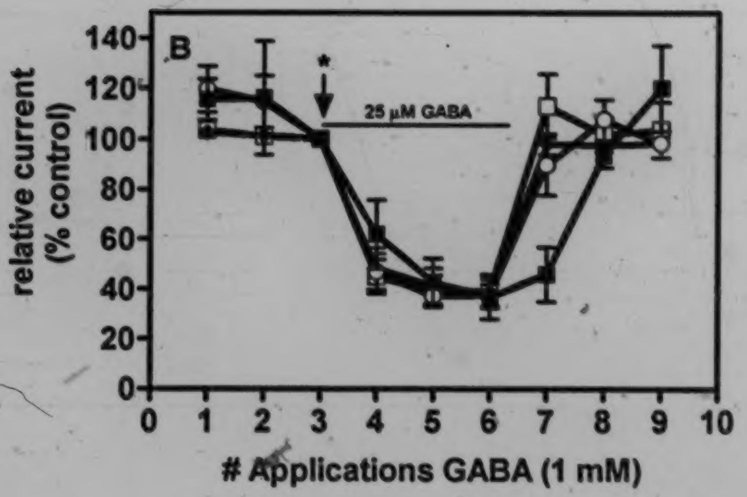
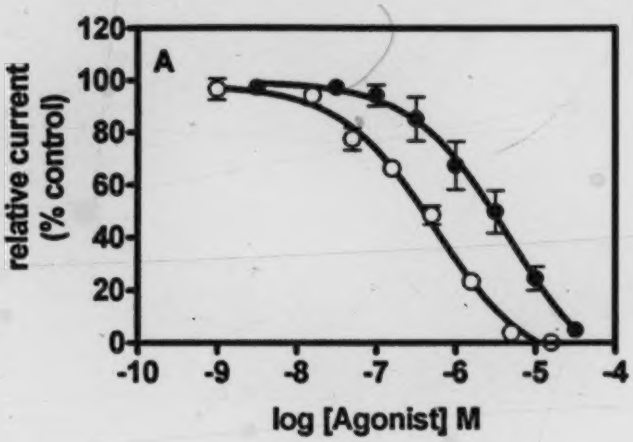
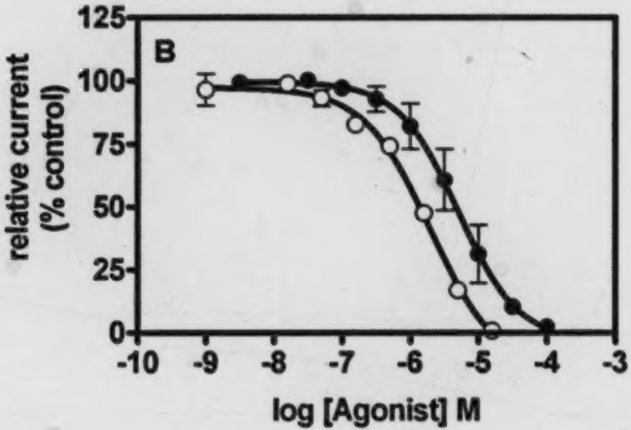

B
*
25 μM GABA
relative current (% control)
# Applications GABA (1 mM)
0
20
40
60
80
100
120
140
0 1 2 3 4 5 6 7 8 9 10

Figure 3-4

Concentration-dependence of agonist-induced desensitization in recombinant $\alpha1\beta2\gamma2$ $GABA_AR$. The response to GABA (1 mM) was stabilized prior to agonist perfusion (see Methods) and this current served as the control value to which all subsequent data were normalized. Data represent the mean current ± SEM (n=3 for muscimol; n=3-6 for GABA) for challenge applications of GABA at each concentration of agonist perfusion. Challenge applications were administered every 12-min (GABA) or 9-min (muscimol) to ensure full recovery from desensitization (see Fig. 3-3). Agonist-mediated current inhibition occurs in a concentration-dependent manner at all mutant receptors for GABA. IC_{50} and n_H values were determined by iterative curve fitting using GraphPad Prism Software. Data for muscimol (○) and GABA (●) at WT (A), Y62F (B), and Y62S (C) receptors are presented in Table 3-1.


A
relative current (% control)
120
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
log [Agonist] M


B
relative current (% control)
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
-3
log [Agonist] M

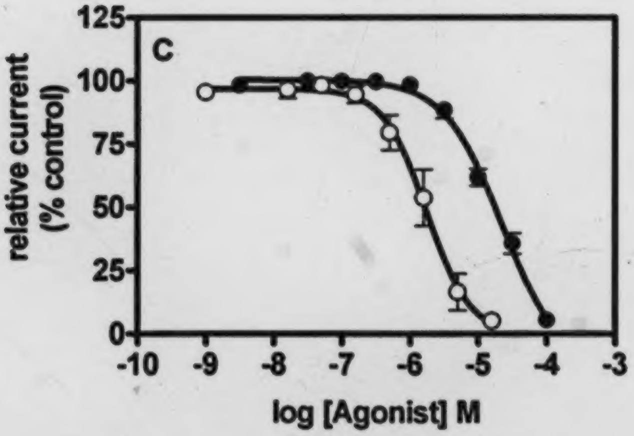

C
relative current (% control)
125
100
75
50
25
0
-10
-9
-8
-7
-6
-5
-4
-3
log [Agonist] M

Figure 3-5

(A) Representative traces from recovery experiments for WT (A) and Y62S (B) containing receptors that are preperfused with agonist at a concentration of 3 μM. At WT receptors, there is no recovery of I_{max} during continuous agonist perfusion. However, receptors expressing Y62S recover to control levels as a function of time even in the presence of GABA.

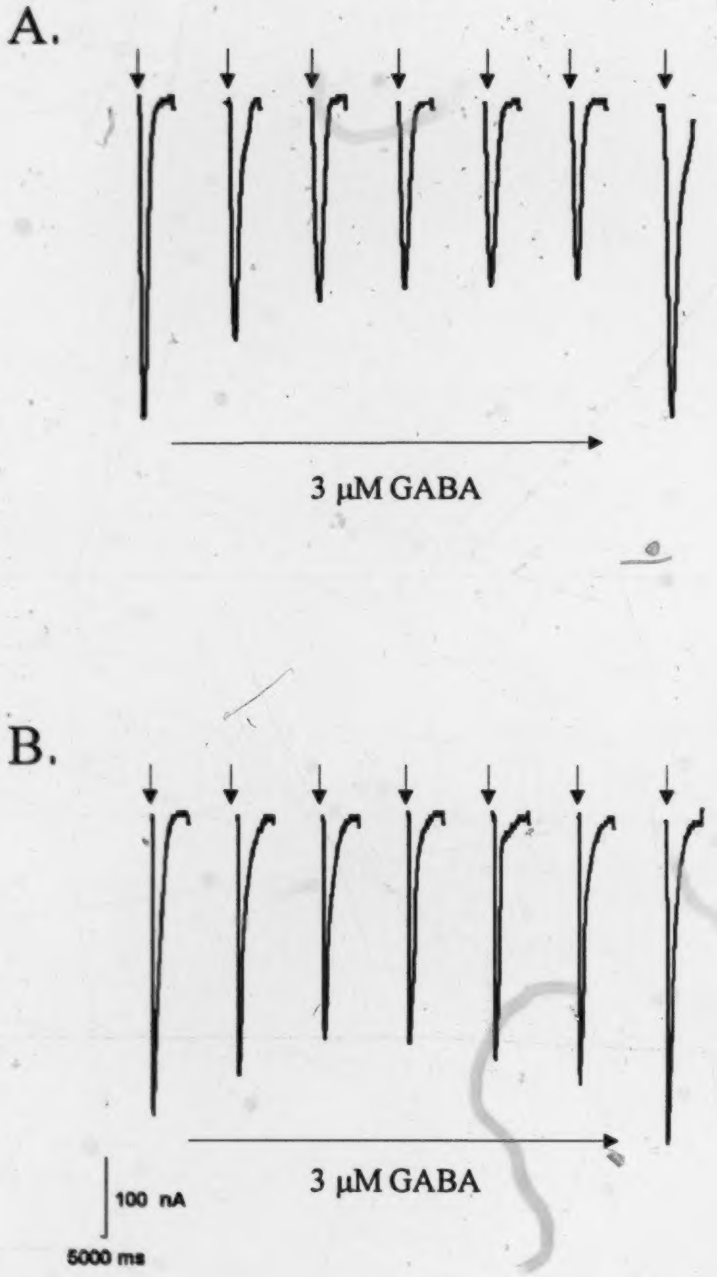

A.
3 µM GABA
B.
3 µM GABA
100 nA
5000 ms

Figure 3-6

Schematic of the allosteric transitions of a recombinant $GABA_AR$ carrying 2 high and 2 low affinity sites. Occupation of low affinity binding sites promotes activation of the receptor, and the transition to the desensitized state is mediated by ligand binding to these same sites. Receptors will proceed to a stabilized desensitized state and recovery will depend on the dissociation of the ligand from these high affinity sites. Also shown are IC_{50} values at each stage of the pathway.

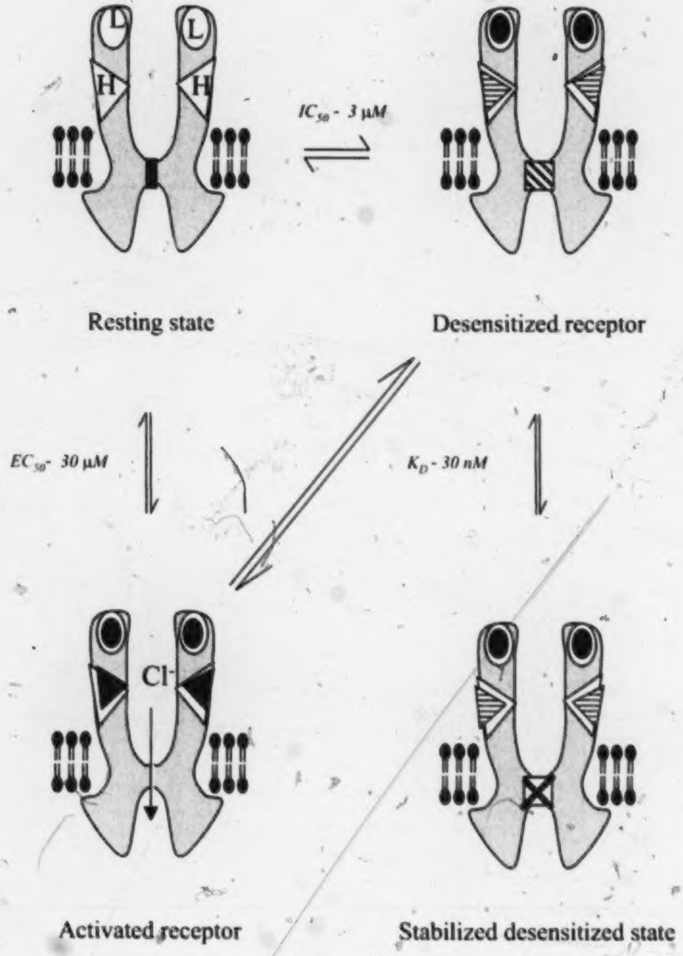

L
L
H
H
IC50 - 3 µM
Resting state
Desensitized receptor
EC50- 30 µM
KD- 30 nM
Cl-
Activated receptor
Stabilized desensitized state

Table 3-1. Effects of β2 subunit mutations on the concentration-dependence of desensitization for muscimol (A) and GABA (B) at recombinant α1β2γ2 $GABA_AR$ expressed in *Xenopus* oocytes. Data represent the mean ± SEM for (n) independent experiments performed as described. Mean log (IC_{50}) values and n_H values were analysed using a one-way analysis of variance (ANOVA) followed by a *post-hoc* Dunnett's test to compare mutants to controls (*P<0.05, **P<0.01). There was no significant difference for the n_H values among all recombinant receptors.

A.

β2 Subunit	Muscimol		
	IC_{50} (μM ± SEM)	n_H	IC_{50} Mutant/IC_{50} WT
WT	0.7 ± 0.01 (3)	-0.77 ± 0.08	1.0
Y62F	2.1 ± 0.3 * (3)	-0.86 ± 0.07	3.2
Y62S	3.8 ± 0.5 ** (3)	-1.32 ± 0.34	5.7

B.

β2 Subunit	GABA		
	IC_{50} (μM ± SEM)	n_H	IC_{50} Mutant/IC_{50} WT
WT	3.3 ± 1.0 (6)	-0.99 ± 0.16	1.0
Y62F	8.2 ± 1.1 * (4)	-1.05 ± 0.07	2.5
Y62S	24.7 ± 5.7 ** (3)	-1.03 ± 0.05	7.5

BIBLIOGRAPHY

AGEY, M.W. AND DUNN, S.M.J. (1989). Kinetics of [^{3}H]muscimol binding to the GABA$_A$ receptor in bovine brain membranes. *Biochemistry*, **28**, 4200-4208.

BARNARD, E.A., SKOLNICK, P., OLSEN, R.W., MÖHLER, H., SIEGHART, W., BIGGIO, G., BRAESTRUP, C., BATESON, A.N. AND LANGER, S.Z. (1998). International union of pharmacology. XV. Subtypes of γ-aminobutyric acid$_A$ receptors: classification on the basis on subunit structure and receptor function. *Pharmacol. Rev.*, **50**, 291-313.

BARTRUP, J.T. AND NEWBERRY, N.R. (1996). Electrophysiological consequences of ligand-binding to the desensitized state of the 5-HT3 receptor in mammalian NG108-15 cells. *J. Physiol.*, **490**, 679-690.

BELLELI, D., CALLACHAN, H., HILL-VENNING, C., PETERS, J.A. AND LAMBERT, J.J. (1996). Interaction of positive allosteric modulators with human and *Drosophila* recombinant GABA receptors expressed in *Xenopus laevis* oocytes. *Br. J. Pharmacol.*, **118**, 563-576.

CASH, D.J. AND SUBBARAO, K. (1987a). Desensitization of γ-aminobutyric acid receptor from rat brain: two distinguishable receptors in the same membrane. *Biochemistry*, **26**, 7556-7562.

CASH, D.J. AND SUBBARAO, K. (1987b). Channel opening of the γ-aminobutyric acid receptor from rat brain: molecular mechanisms of the receptor response. *Biochemistry*, **26**, 7562-7570.

CLEMENTS, J.D. (1996). Transmitter tinecourse in the synaptic cleft: its role in central synaptic function. *Trends Neurosci.*, **19**, 163-171.

CORRINGER, P.-J., BERTRAND, S., BOHLER, S., EDELSTEIN, S.J., CHANGEUX, J.-P. AND BERTRAND, D. (1998). Critical elements determining diversity in agonist binding and desensitization of neuronal nicotinic acetylcholine receptors. *J Neurosci*, **18**, 648-657.

DESTEXHE, A. AND SEJNOWSKI, T.J. (1995). G protein activation kinetics and spillover of γ-aminobutyric acid activation may account for differences between inhibitory responses in the hippocampus and amygdala. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 9515-9519.

DAVIES, M., STEELE, J.A., HADINGHAM, K.L., WHITING, P.J. AND DUNN, S.M.J. (1994). Characterization of a permanently transfected cell line expressing the human $\alpha 1 \beta 1 \gamma 2S$ $GABA_A$ receptor subtype. *Can. J. Physiol. Pharmacol.*, **72**, 337.

DUNN, S.M.J., BATESON, A.N. AND MARTIN, I.L. (1994). Molecular neurobiology of the GABAA receptor. *Int. Rev. Neurobiol.*, **35**, 51-96.

DUNN, S.M.J. AND RAFTERY M.A. (1982). Multiple binding sites for agonists on Torpedo californica acetylcholine receptor. *Biochemistry*, **21**, 6264-6272.

EDMONDS, B., GIBB, A.J. AND COLQUHOUN, D. (1995). Mechanisms of activation of muscle nicotinic acetylcholine receptors and time course of endplate currents. *Ann. Rev. Physiol.*, **57**, 495-519.

HUGANIR, R.L. AND GREENGARD, P. (1990). Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron*, **5**, 555-567.

HUANG, E.P. (1998). Synaptic transmission: spillover at central synapses. *Curr. Biol.* **8**, R613-R615.

JONES, M.W. AND WESTBROOK, G.L. (1995). Desensitized states prolong $GABA_A$ channels to brief agonist pulses. *Neuron*, **15**, 181-191.

JONES, M.V., SAHARA, Y., DZUBAY, J.A., AND WESTBROOK, G.L. (1998). Defining affinity with the $GABA_A$ receptor. *J. Neurosci.*, **18**, 5590-5604.

KUHLMANN, J., OKONJO, K.O., AND MAELICKE, A. (1991). Desensitization is a property of the cholinergic binding region of the nicotinic acetylcholine receptor, not of the receptor-integral ion channel. *FEBS Lett.*, **279**, 216-218.

LERMA, J., HEIRANZ, A.S., HERRERS, V., ABRAIRA, V., AND MARTIN DEL RIO, R. (1986). In vivo determination of extracellular concentration of amino acids in the rat hippocampus: a method based on brain dialysis and computerized analysis. *Brain. Res.*, **384**, 145-155.

LOHSE, M.J. (1993). Molecular mechanisms of membrane receptor desensitization. *Biochim. Biophys. Acta.*, **1179**, 171-188.

MONOD, J., WYMAN, J. AND CHNAGEUX, J.-P. (1965). On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.*, **12**, 88-118.

NEWELL, J.G., DAVIES, M., BATESON, A.N. AND DUNN S.M.J. (2000a). Tyrosine of the γ-aminobutyric acid type A receptor β2 subunit is an important determinant of high affinity agonist binding. *J. Biol. Chem.*, **275**, 14198-14204.

NEWELL, J.G., DAVIES, M. AND BATESON, A.N. (2000b). The use of site-directed mutagenesis, transient transfection and radioligand binding: a method for the characterization of receptor-ligand interactions. *Mol. Biotech.*, **14**, 25-45.

OCHOA, E.R.M., CHALLOPADHYAY, A. AND MCNAMEE, M.G. (1989). Desensitization of the nicotinic acetylcholine receptor: molecular mechanisms and effects of modulators. *Cell & Mol. Neurobiol.*, **9**, 141-177.

OLSEN, R.W., BERGMAN, M.O., VAN NESS, P.C., LUMMIS, S.C., WATKINS, A.E., NAPIAS, C. AND GREENLEE, D.V. (1981). γ-Aminobutyric acid receptor binding in mammalian brain: heterogeneity of binding sites. *Mol. Pharmacol.*, **19**, 217-227.

RAFTERY, M.A., DUNN, S.M.J., CONTI-TRONCONI, B.M., MIDDLEMAS, D.S. AND CRAWFORD, R.D. (1983). The nicotinic acetylcholine receptor: subunit structure, functional binding sites, and ion transport properties. *Cold Harb. Symp. Quant. Biol.*, **48**, 21-33.

SCHOFIELD, P.R., DARLISON, M.G., FUJITA, N., BURT, D.R., STEPHENSON, F.A., RODRIGUEZ, H., RHEE, L.M., RAMACHANDRAN, J., REALE, V., GLENCORSE, T.A., SEEBURG, P.H. AND BARNARD, E.A. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature*, **328**, 221-227

SHANK, R.P., BALDY, W.J., MATTUCCI, L.C., AND VILLANI, F.J. JR. (1990). Ion and temperature effects on the binding of γ-aminobutyrate to its receptors and the high affinity transport system. *J. Neurochem.*, **54**, 2008-2015.

YANG, J.S.-J. AND OLSEN, R.W. (1987). γ-Aminobutyric acid receptor binding in fresh mouse brain membranes at 22°C: ligand-induced changes in affinity. *Mol. Pharmacol.*, **32**, 266-277.

CHAPTER 4[1]

Mutagenesis Of The GABA$_A$ Receptor α1 Subunit Reveals A Domain That Affects Sensitivity To GABA And Benzodiazepine Site Ligands

---

[1] *A version of this chapter has been submitted for publication.* DAVIES, M., NEWELL, J.G., AND DUNN, S.M.J. *J. Neurochem.* Dr. Martin Davies engineered all mutants and collected all radioligand binding data.

INTRODUCTION

γ-Aminobutyric type A ($GABA_A$) receptors are a group of heterogeneous ligand-gated ion channels that are found throughout the mammalian CNS. Their heterogeneity arises from the large number of subunits that can potentially be incorporated into any particular receptor. Several subunit-encoding genes have been identified, including α1–6, β1–3, γ1–3, ρ1–3, δ, ε, π and θ (for reviews, *see* Barnard *et al.*, 1998; Bonnert *et al.*, 1999). The subunit complement of a receptor determines its pharmacological profile, and it is known that for a $GABA_A$ receptor to be sensitive to classical benzodiazepines, it must contain specific α (1, 2, 3, or 5) and γ (1, 2 or 3) subunits (*see* Sieghart, 1995). It is thought that the benzodiazepine binding domain lies at the interface between the α and γ subunits (Sigel and Buhr, 1997), and several amino acids in these subunits have been shown to participate in the formation of this site (Pritchett and Seeburg, 1991; Wieland *et al.*, 1992; Mihic *et al.*, 1994 Buhr *et al.*, 1996; Amin *et al.*, 1997; Renard *et al.*, 1999). Similarly, GABA recognition domains are thought to be formed by homologous regions of other subunits lying at the β-α interface (Sigel *et al.*, 1992; Amin and Weiss, 1993). In both cases, amino acids involved in ligand recognition appear to be clustered in specific regions or "loops" in the N-terminal domains of these subunits. This pattern of amino acid clusters is also seen in the ligand recognition domains of the prototypical ligand-gated ion channel, the nicotinic acetylcholine receptor (Galzi and Changeux, 1995), and it has been proposed that these binding motifs are shared by all members of this receptor family (Sigel and Buhr, 1997).

Photoaffinity labeling studies have shown that the agonist flunitrazepam and the inverse agonist Ro15-4513 covalently label different regions of the α1 subunit (Davies *et al.*, 1996). Labeling with [^{3}H]flunitrazepam results in incorporation of the label into a domain N-terminal to residues 102/103, whereas the site(s) of [^{3}H]Ro15-4513 photolabelling is C-terminal to these residues. More detailed studies have shown that the major site of [^{3}H]flunitrazepam labelling is His-101 (Duncalfe *et al.*, 1996) of the α1 subunit, whereas [^{3}H]Ro15-4513 labels a domain that encompasses TMII and the small extracellular loop between TMII and TMIII (Davies and Dunn, 1998), a domain that has also been implicated in forming the recognition sites for ethanol, anesthetics (Mihic *et al.*, 1997) and divalent cations (Fisher and MacDonald, 1998). A recent study demonstrated that a lysine residue (K278) in this loop of the α1 subunit plays a role in determining GABA sensitivity (Sigel *et al.*, 1999). Additionally, a study of the ρ1 receptor showed that mutation of amino acids in this region could completely abolish GABA recognition (Kusama *et al.*, 1994). In the present study, we have introduced a series of point mutations in the putative extracellular loop between TMII and TMIII of the α1 subunit and have examined the effects of these mutations on both GABA and benzodiazepine site ligand sensitivity.

MATERIALS AND METHODS

Site-Directed Mutagenesis

Rat cDNAs encoding the α1, β2 and γ2 subunits were kindly provided by Dr. D.S. Weiss. Mutagenesis was performed using either the Altered Sites® *in vitro* Mutagenesis System (Promega Corporation, Madison, WI, USA) or Gene Editor (Promega Corporation) kits. The targets for mutagenesis are illustrated in Fig. 4-1. Mutagenic oligonucleotides were synthesized to introduce both the desired mutation and a silent restriction site for use in the preliminary screening of putative mutants (*see* Davies *et al.*, 1998). The primers used are listed below.

S275T, 5' ATAAGCCACCTTAGGGAGGGTATTTCTGGCACT 3'
L276A, 5' CATAAGCCACCTTAGGGGCGGAATTTCTGGC 3'
P277A, 5' CATAAGCCACCTTCGCGAGGGAATTTCTGGC 3'
V279A, 5' CGTTGCATAAGCGGCCTTAGGGAGGGAATTTCTG 3'
A280S, 5' GGCCGTTGCATAAGACACCTTAGGGAGGGAATTTCT 3'
Y281F, 5' CATGGCCGTTGCAAAAGCCACCTTAGGGAGGGAATTTCT 3'

Mutations were verified by sequencing and the mutated cDNA was then subcloned into the pcDNA3.1(+) mammalian expression vector (Invitrogen).

Cell Culture and Transient Transfection

tsA201 cells were grown in Dulbecco's low glucose modified media which was supplemented with 10% Fetal Clone III (Hyclone) and 100 U/ml penicillin-streptomycin solution. Transient transfection was performed as described previously using 10 μg of each subunit construct per 15 cm dish (Davies *et al.*, 1998). After 48 hours, the cells were harvested in 50 mM Tris-HCl buffer containing protease inhibitors and stored at -80°C until used in binding assays.

Equilibrium Binding Assays

Binding assays were performed by filtration assays as previously described (Davies *et al.*, 1998). [^{3}H]Ro15-4513 was used over a concentration range of 1- 60 nM and non-specific binding was determined in the presence of 10 μM flunitrazepam. For

[^{3}H]EBOB (4'–ethynyl-4-n-propylbicycloorthobenzoate) binding, membranes were incubated in the presence of the radioligand at room temperature for 120 min. Non-specific binding was determined in the presence of 200 μM picrotoxin. The filters used for [^{3}H]EBOB binding experiments were soaked in buffer containing 0.3% polyethylenimine.

Expression in Oocytes and Two-Electrode Voltage Clamp Analysis

GABA$_A$ receptor subunit cDNAs were transcribed as described previously (Hope *et al.*, 1993) and 50 ng total of subunit cRNA (with the subunits in a 1:1:1 ratio) was injected into *Xenopus laevis* oocytes (Belelli *et al.*, 1996) which were maintained for 2-10 days in modified Barth's medium [(in mM) NaCl (88), KCl (1), $CaCl_2$ (0.5), $Ca(NO_3)_2$ (0.5), sodium pyruvate (2.5), $MgSO_4$ (1), $NaHCO_3$ (2.4), 4-(2-(hydroxyethyl)-1-piperazinethanesulonic acid (HEPES) (15), pH 7.4, supplemented with gentamicin, 100μg/ml]. Standard two-electrode voltage clamp was carried out using a GeneClamp amplifier (Axon Instruments Inc.), and currents were recorded using pClamp 6 software (Axon Instruments Inc.). Electrodes were filled with 3M KCl and had resistances of 0.5 – 2.0 MΩ in frog Ringer's. The holding potential was –60 mV and oocytes were continually perfused with frog Ringer's solution [(in mM) NaCl (120), HEPES (5), KCl (2) and $CaCl_2$ (1.8)]. Voltage clamp procedures were carried out using a GeneClamp 500 amplifier (Axon Instruments Inc.) Drugs were applied via a gravity perfusion system with a 3-15 min wash out between applications. For experiments with allosteric modulators, GABA, at a concentration equivalent to its EC_{10}, was used for flunitrazepam potentiation studies whereas the EC_{50} concentration was used in experiments with Ro15-4513. In each case the benzodiazepine site ligand was pre-perfused for 3 min. to reach equilibrium before addition of GABA and the same concentration of benzodiazepine. Stock solutions of benzodiazepine site ligands were made in DMSO and the final DMSO concentration in all solutions was constant at 0.03%.

Data and Statistical Analyses

Saturation binding and concentration response curves were analyzed by non-linear regression using GraphPad Prism. Data were fit by equations as described in

Chapters 2 and 5. Statistical analysis was performed with the statistical package included with the GraphPad Prism software as detailed in the text.

RESULTS

Equilibrium Binding Assays

Binding experiments were carried out using membranes prepared from tsA201 cells transfected with either wild-type or mutant α subunits together with β2 and γ2 constructs. The direct binding of [^{3}H]Ro15-4513 was investigated since previous work has indicated that the domain mutated in the present study lies in a region that is photoaffinity labeled by this ligand (Davies and Dunn, 1998). Except for receptors containing the V279A mutation, the binding affinity for [^{3}H]Ro15-4513 was unaffected by the substitutions (Table 4-1). Of the receptor preparations that recognized [^{3}H]Ro15-4513, all showed similar binding capacities (data not shown), indicating that the mutations did not drastically affect receptor assembly and transportation to the cell surface. Some variability in expression levels among different batches of cells is, however, inevitable due to differences in transfection efficiencies. In the case of the V279A mutant receptor, the dramatic reduction in affinity for [^{3}H]Ro15-4513 (Table 4-1) precluded the use of this ligand to quantitate receptor densities. No specific binding was measurable using [^{3}H]Ro15-1788 (2 nM) or [^{3}H]flunitrazepam (10 nM), suggesting that the mutation had a general detrimental effect on the binding of benzodiazepine site ligands. To verify that the V279A mutant was indeed expressed on the cell surface, [^{3}H]EBOB binding experiments were carried out and its binding to the mutant was compared with binding to the wild-type receptor. This radioligand binds to the picrotoxin site of GABA$_A$ receptors (Kume and Albin, 1994). At a concentration of 2.5 nM, there was no observable difference in [^{3}H]EBOB binding between wild-type and V279A receptors (0.64 ± 0.07 and 0.51 ± 0.07 pmol/mg protein, respectively). Thus the V279A mutation does not adversely affect the assembly of the receptor.

Two-Electrode Voltage Clamp Analysis

The functional consequences of the substitutions in the α1 subunit were determined using electrophysiological techniques. Coexpression of the mutated α1 subunits with β2 and γ2 subunits in *Xenopus* oocytes resulted in receptors that were gated by GABA (Figs. 4-2, 4-3, 4-4). However, while certain mutations showed no apparent change in GABA response, others resulted in receptors that displayed a rightward shift in the concentration-response curves for GABA (Fig. 4-2).

The mutations S275T, A280S and Y281F had no apparent effect on the concentration-response curves of the receptors to GABA, and estimated EC_{50} values were not significantly different from that of wild-type receptors. In contrast, the L276A, P277A, and V279A mutations resulted in receptors that were less sensitive to GABA. The alanine substitutions at positions 277, 279, and 280 produced similar effects with an increase in the EC_{50} value of approximately two-fold while the alanine substitution at position 276 produced the largest change in the GABA concentration dependence with a rightward shift of approximately 6-fold. None of the Hill coefficients was significantly different from the wild type value (Table 4-2).

As described above, the only difference in [^{3}H]Ro15-4513 binding was found with the V279A mutant, and the functional consequences of this mutation were, therefore, examined electrophysiologically. Flunitrazepam potentiated the GABA-gated current with an EC_{50} of 122 nM (Fig. 4-5A) that is approximately 20-fold greater than its EC_{50} for potentiation of wild-type receptors (Table 4-3). At high concentrations of flunitrazepam, the maximum potentiation observed with the V279A mutant was comparable to that of wild-type receptors (Fig. 4-5A; Fig. 4-6).

In studies of the functional effects of Ro15-4513 on GABA-gated currents, this ligand was found to retain its inverse agonist properties at the V279A mutant (Fig 4-5B), but the EC_{50} (40 nM) for this effect was 22-fold greater than for wild-type (Table 4-3). Ro15-1788 at a concentration of 100 nM had no direct effect on GABA-induced currents in the V279A receptor, suggesting that it remained an antagonist (Fig. 4-3). An estimated affinity for this ligand was, therefore, obtained from its ability to inhibit the flunitrazepam potentiating effect (Fig. 4-5C; Fig. 4-6). The K_I value was approximately 4-fold higher than for the wild-type receptor measured under similar experimental conditions (Table 4-3).

To determine if the functional coupling between flunitrazepam and GABA sites had been altered in the V279A mutant, GABA concentration-response curves for wild-type and the mutant were carried out in the presence of saturating concentrations of flunitrazepam (100 nM and 2 μM, respectively). In the presence of flunitrazepam both receptor concentration-response curves were shifted to the left to an equivalent degree (Fig. 4-7).

DISCUSSION

This study describes a residue, V279 of the α1 subunit, which affects the sensitivity of $GABA_A$ receptors to benzodiazepine site ligands. Previous studies from this laboratory indicated that the photolabel, [^{3}H]Ro15-4513, was covalently incorporated into a domain that includes the putative extracellular loop between TMII and TMIII of the $GABA_A$ receptor α1 subunit (Davies and Dunn, 1998). To define further the location of this putative Ro15-4513 binding site, we made a series of mutations within this loop. The substitutions (Fig. 4-1) were chosen for several reasons. Firstly, the residues in this domain are fairly well conserved in all α subunits with the exception of α4 and α6, which contain a histidine instead of an arginine at position 274 and a serine instead of an alanine at position 280. Since receptors containing α4 and α6 subunits are insensitive to "classical" benzodiazepines such as flunitrazepam, the possible involvement of these residues in benzodiazepine recognition was investigated by substituting the α1 residues in these positions to those found in α4 and α6 (A280S). Other residues were replaced with conservative substitutions, in order to minimize changes that could potentially disrupt the overall structure of the receptor. One exception to this was the proline to alanine substitution at position 277. Proline is considered a "helix breaker" and we, therefore, predicted that substitution of an alanine at this position could perturb the secondary structure of this region.

All the mutations were tolerated. The results from radioligand binding experiments with all the mutants except V279A showed K_D values for [^{3}H]Ro15-4513 binding that were similar to wild type, indicating that the overall receptor conformation was not drastically altered. The results of the binding experiments with [^{3}H]EBOB showed that, although the V279A mutant displayed a reduction in benzodiazepine site ligand binding, the mutant receptor expressed on the cell surface at a level that was comparable to the wild-type receptor. Thus the observed decrease in binding of benzodiazepine site ligands is due to a loss of recognition rather than a decrease in receptor expression.

For some of the mutations, the changes in GABA-gated currents were unexpected since the present study was initiated to examine an apparently novel binding domain for Ro15-4513. However, during the writing of this manuscript, the results of a similar study from another laboratory (Sigel *et al.*, 1999) confirmed that the agonist response of $GABA_A$ receptors is sensitive to changes of K278 in this domain in both α1 and β2 subunits, but not in the γ2 subunit. However, no effects on benzodiazepine sensitivity were reported. The results in the present study, together with those of Sigel *et al.* (1999) show that this loop contains several amino acids important in determining GABA sensitivity and that they are clustered together in a contiguous domain at positions L276, P277, K278, and V279 (see Fig. 4-1 and Table 4-2).

In general, the data arising from mutations of ligand-gated ion channels must be interpreted with caution (Colquhoun, 1998). A shift in EC_{50} values for agonists does not necessarily indicate that the binding site for GABA has been altered; it is equally likely that the gating of the receptor has been altered in some fashion. Indeed, the latter has been suggested to be responsible for the change in GABA sensitivity that was seen when mutations were introduced at position 271 in this region (Sigel *et al.*, 1999). A similar study using ρ receptors showed that a single mutation in this loop produced GABA-concentration response curves with an increased Hill coefficient of 3.9 (Kusama *et al.*, 1994). Again, the authors suggest that this indicates that the mutation affects channel gating. Consistent with these conclusions are data from other mutagenic studies suggesting that the binding domains for GABA are found in the β subunits and in the α subunit N-terminus at a site distant from the one mutated here (Sigel *et al.*, 1992; Amin and Weiss, 1993). Additionally, mutations in this general region of the glycine (Langosch *et al.*, 1994) and neuronal nicotinic receptor (Campos-Cara *et al.*, 1996) are thought to affect the coupling of the agonist binding event to the opening of the channel. Only further studies at the single channel level will allow the effects of the mutations on binding and gating to be distinguished.

The V279A mutant was unique in that it resulted in a large increase in the EC_{50} values for the effects of benzodiazepine site ligands on GABA-gated currents. The EC_{50} for potentiation of currents by flunitrazepam was increased approximately twenty-fold but this ligand remained a full agonist and the maximum level of potentiation was not significantly different from wild-type. In the presence of saturating concentrations of flunitrazepam, the concentration dependencies of GABA-activated currents were affected to a similar extent (Fig. 4-7). Together, these results suggest the allosteric coupling between the flunitrazepam and GABA sites is retained in the V279A mutant receptor. The apparent affinities for Ro15-4513 and Ro15-1788 were also reduced as a result of this mutation, suggesting that the recognition of all benzodiazepine site ligands may have been compromised. However, the efficacy of these ligands was unaffected since Ro15-4513 and Ro15-1788 remained an inverse agonist and antagonist, respectively. This indicates that the type of conformational change induced by these ligands has not been adversely affected by the mutation.

The region chosen for mutation is the putative site of photolabeling by [^{3}H]Ro15-4513 (Davies and Dunn, 1998). This suggests, therefore that this domain may be part of a binding site for this ligand, or lies in close proximity to such a site. The large reduction in affinity for flunitrazepam as a result of the conservative V279A mutation was not anticipated since this ligand has been reported to label a residue, histidine 101 (Duncalfe *et al.*, 1996) that lies in a domain homologous to those predicted to form agonist binding sites in other receptors (Sigel and Buhr, 1997). In the nicotinic acetylcholine receptor, these agonist sites are postulated to lie approximately 30 Å above the membrane surface (Unwin, 1993) i.e. physically distant from the small extracellular loop mutated in this report. Further structural information is required to distinguish between a direct involvement of V279 in the binding site or as part of the transduction process mediated by the binding of benzodiazepine site ligands.

In conclusion, this study illustrates the importance of a four amino acid domain in the extracellular loop between TMII and TMIII of the GABA$_A$ receptor $\alpha 1$ subunit in determining the sensitivity of the receptor to both GABA and benzodiazepine site ligands.

Figure 4-1

Residues 273 to 283 in the putative extracellular loop between TMII and TMIII of the rat $\alpha 1$ subunit. Wild-type residues are shown in the upper strand of amino acids. The arrows indicate the substitution that was introduced at specific residues. The dashed arrow represents an amino acid substitution made by Sigel *et al.* (1999), where X represents E, Q and R.

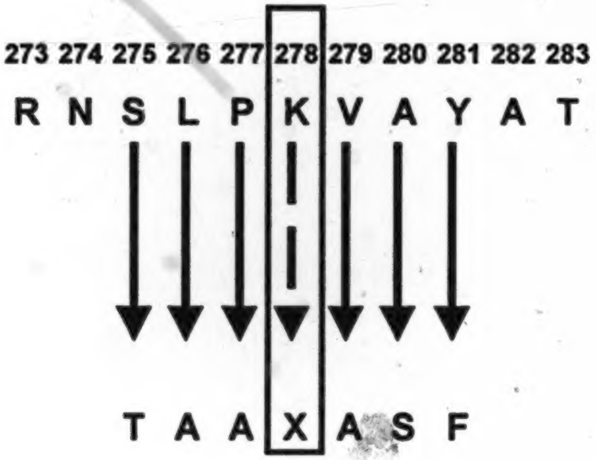

273 274 275 276 277 278 279 280 281 282 283
R N S L P K V A Y A T
T A A X A S F

Figure 4-2

Concentration response relationships for GABA with wild-type (●) and mutant receptors S275T (○), L276A (◆), P277A (■), V279A (□), A280S (Δ) and Y281F (+). Data represent the mean ± SEM for 3-5 independent experiments. Data for all recombinant receptors are summarized in Table 4-2.

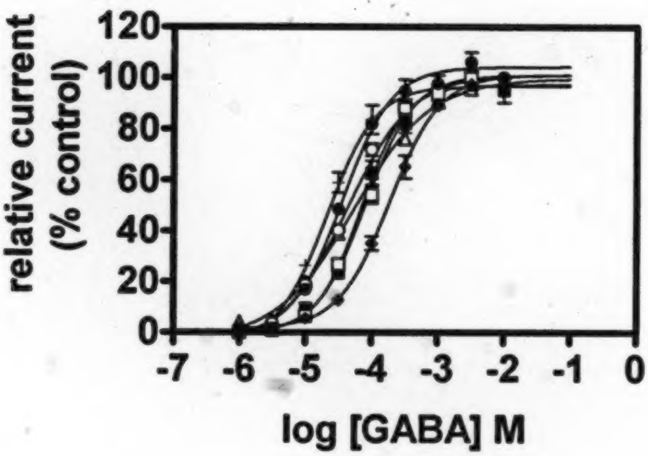

relative current
(% control)
120
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
-1
0
log [GABA] M

Figure 4-3

Representative traces showing GABA activation of recombinant $GABA_AR$ expressing WT (A), S275T (B), L276A (C) and P277A (D) $\alpha 1$ subunits.

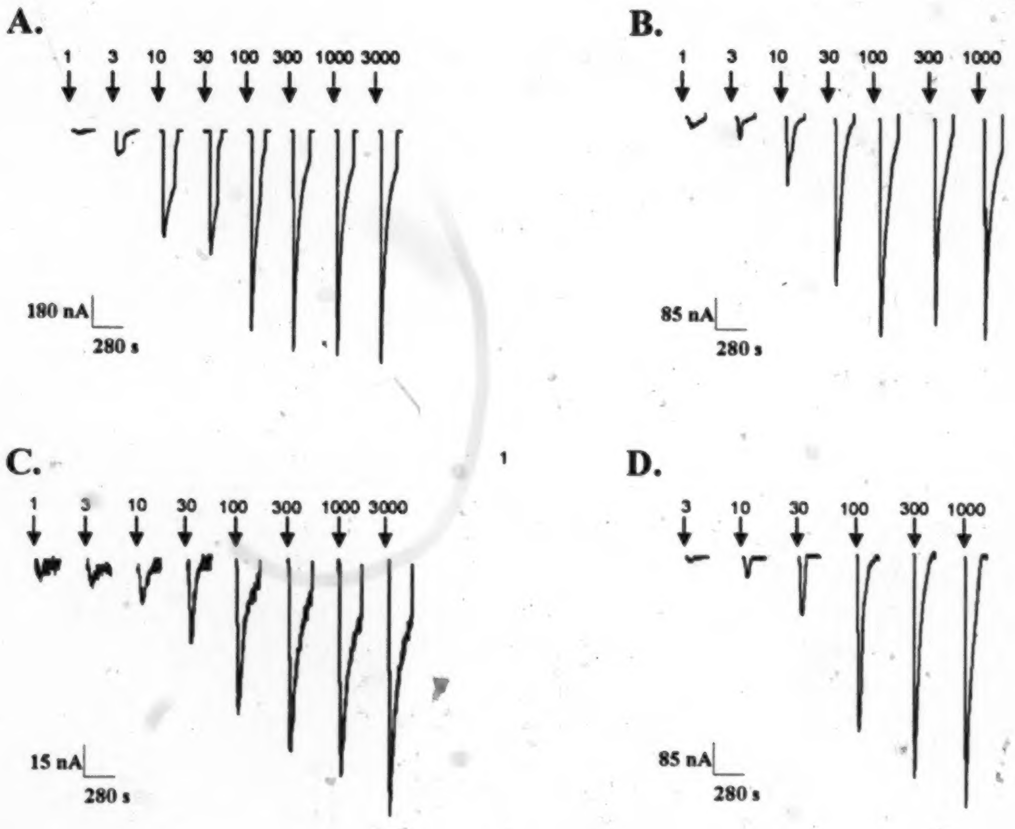

A.
1
3
10
30
100
300
1000
3000
180 nA
280 s
B.
1
3
10
30
100
300
1000
85 nA
280 s
C.
1
3
10
30
100
300
1000
3000
15 nA
280 s
D.
3
10
30
100
300
1000
85 nA
280 s

Figure 4-4

Representative traces showing GABA activation of recombinant $GABA_AR$ expressing V279A (A), A280S (B) and Y281F (C) $\alpha1$ subunits.

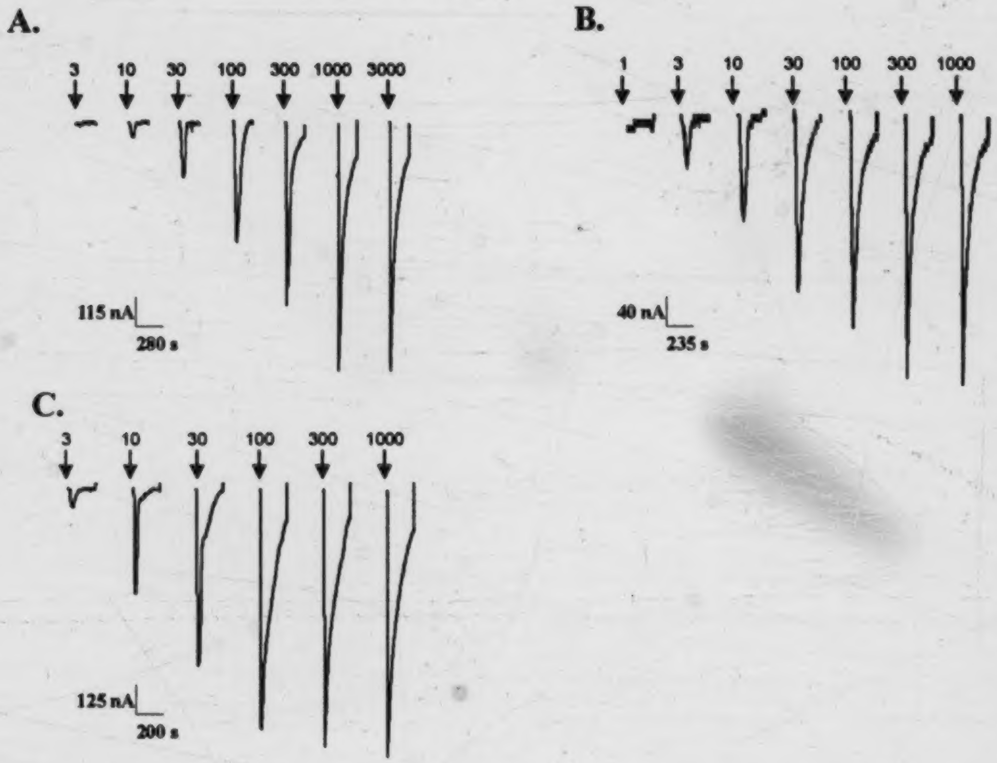

A.
3
10
30
100
300
1000
3000
115 nA
280 s
B.
1
3
10
30
100
300
1000
40 nA
235 s
C.
3
10
30
100
300
1000
125 nA
200 s

Figure 4-5

The V279A mutation does not change the efficacy of benzodiazepine site ligands. A) Flunitrazepam concentration-response relationships for receptors containing α subunits with the V279A mutation. The EC_{50} for potentiation of GABA-gated (15 μM) chloride flux is 122.2 ± 6.0 nM and the E_{max} is 375 ± 65% ($n_H = 0.92 \pm 0.12$). B) Ro15-4513 remains an inverse agonist at the V279A mutant, but with reduced potency. GABA was present at the EC_{50} concentration for these experiments. The IC_{50} for Ro15-4513 was 40.1 ± 8.4 nM ($n_H = -2.4 \pm 0.9$). C) Ro15-1788 acts as an antagonist at V279A receptors. Oocytes were pre-perfused with 100 nM flunitrazepam. GABA (at the EC_{10}) and 100 nM flunitrazepam were then added in concert to establish the maximum potentiation. Subsequent to this, responses were determined in the presence of GABA, flunitrazepam and increasing concentrations of Ro15-1788. The resulting IC_{50} for the antagonism of the potentiating effects of flunitrazepam (100 nM) by Ro15-1788 was 33.6 ± 6.8 nM ($n_H = -1.04 \pm 0.09$). For display purposes only, data were normalized by setting the maximum potentiation as 100% and maximum inhibition as baseline (GABA-gated current in the absence of flunitrazepam).

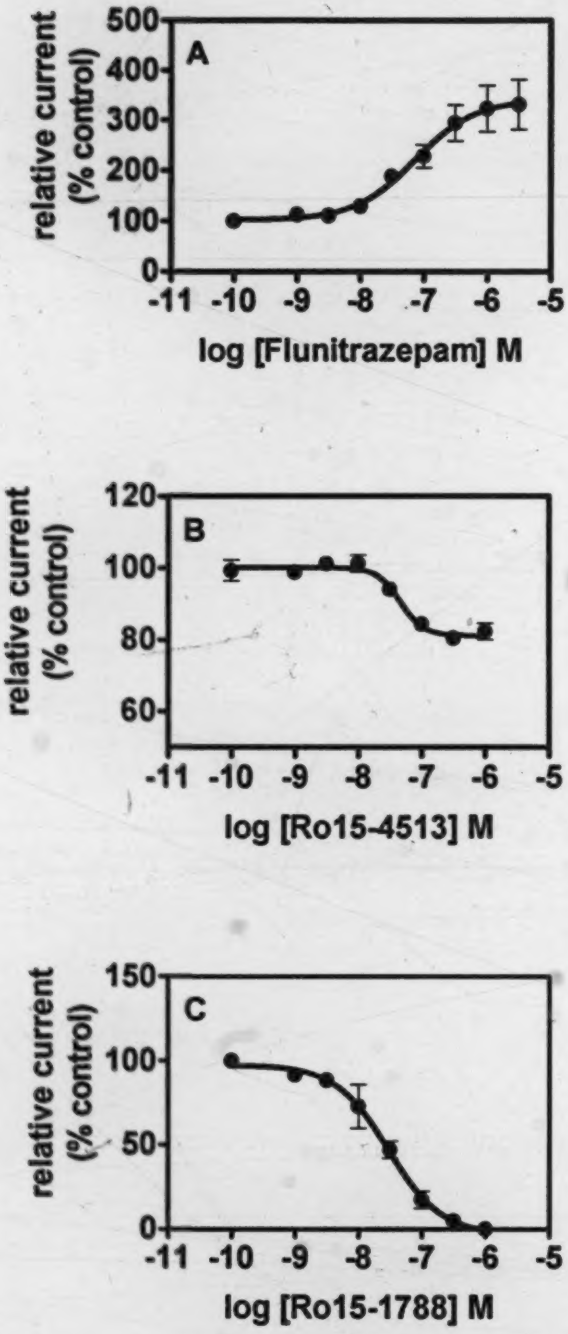

A
relative current (% control)
500
400
300
200
100
0
-11
-10
-9
-8
-7
-6
-5
log [Flunitrazepam] M
B
relative current (% control)
120
100
80
60
-11
-10
-9
-8
-7
-6
-5
log [Ro15-4513] M
C
relative current (% control)
150
100
50
0
-11
-10
-9
-8
-7
-6
-5
log [Ro15-1788] M

Figure 4-6

Representative traces for the V279A mutation illustrating the pharmacological actions of benzodiazepines ligands on GABA-gated current. A) Flunitrazepam potentiates (400%) the effects of GABA (15 μM). This potentiation is blocked by Ro15-1788 (100 nM) and the current resulting from the application of both FNZ and Ro15-1788 is 28% greater than control. B). Application of Ro15-4513 (1 μM) reduces GABA (80 μM) gated-current by ~20%.

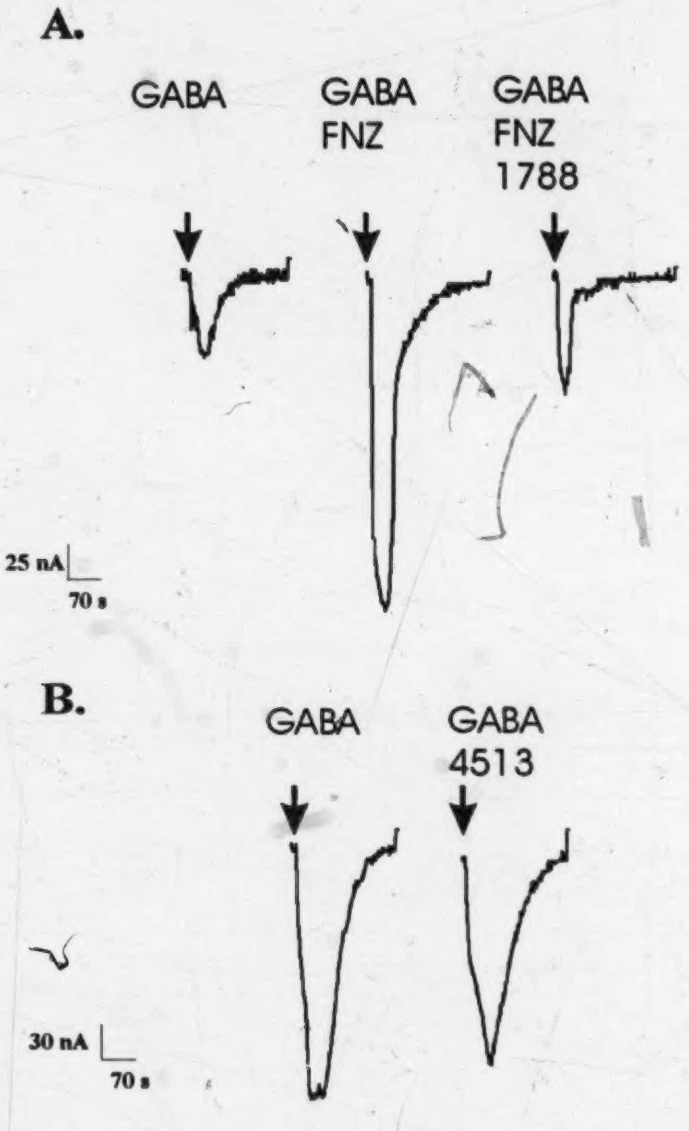

A.
GABA
GABA
FNZ
GABA
FNZ
1788
25 nA
70 s
B.
GABA
GABA
4513
30 nA
70 s

Figure 4-7

The effects of flunitrazepam on GABA concentration-response curves for wild-type and V279A receptors. The concentration response relationships for wild-type and V279A receptors were compared in the absence (open squares and circles, respectively) and presence of flunitrazepam (closed squares and circles, respectively). A concentration that brought about a maximal potentiation of GABA-gated current for both receptor types was used (100 nM for wild type and 2 μM for V279A). Flunitrazepam resulted in a significant shift in the curve to the left for wild-type and V279A receptors ($P< 0.01$) (EC_{50} values of 11.6 ± 0.8 and 40.5 ± 7.7 nM, respectively). The ratio of EC_{50} values in the presence and absence of flunitrazepam was 2.8 ± 0.2 and 2.2 ± 0.5 for wild-type and V279A receptors, respectively. The Hill slopes for GABA-mediated chloride conductance in the presence of flunitrazepam for receptors in which the wild-type $\alpha 1$ and V279A $\alpha 1$ mutant subunit were expressed were 1.37 ± 0.02 and 1.06 ± 0.13, respectively.

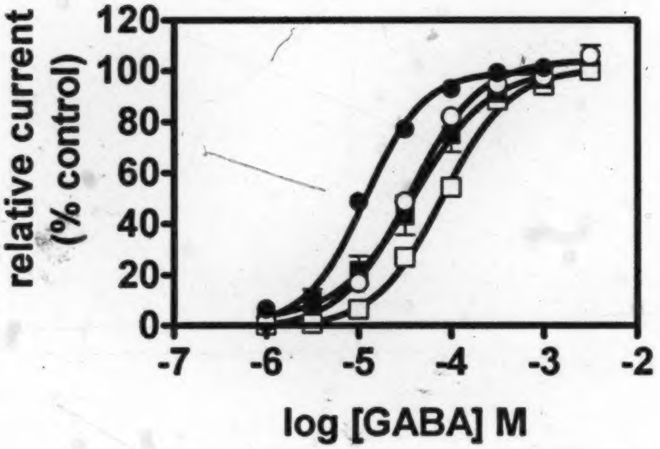

relative current
(% control)
120
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
log [GABA] M

Table 4-1. The affinity of [^{3}H]Ro15-4513 for wild-type receptors compared to those containing mutated α1 subunits. Values represent mean ± SEM for at least two experiments performed in duplicate. No significant difference was observed for the K_D values of the Ro15-4513 binding sites.

α-subunit	K_D (nM± SEM)
wild-type	9.5 ± 0.1
S275T	9.2 ± 3.0
L276A	6.4 ± 1.5
P277A	8.5 ± 2.7
V279A	no high affinity binding*
A280S	7.9 ± 1.7
Y281F	7.8 ± 2.0

*There was no detectable high affinity binding at low concentrations of [^{3}H]Ro15-4513. Some specific binding was detectable at higher concentrations (> 20 nM), but because of the decreased affinity of the receptor for this ligand, it was not feasible to perform complete saturation binding experiments.

Table 4-2. Concentration-response data for GABA-mediated chloride flux from recombinant GABA$_A$ receptors expressed in *Xenopus* oocytes. Data represent the mean ± SEM for (n) experiments performed in duplicate. Log (EC$_{50}$) and n_H values analyzed by one-way ANOVA, followed by a post-hoc Dunnett's test to determine levels of significance (*P<0.01). Hill slopes (n_H) were not significantly different from controls. $^{\&}$From Newell *et al.* (2000). The mean values were calculated by using the parameters obtained from each experiments on individual oocytes (>5 batches) that had been injected using 2-3 different batches of cRNA.

α1 subunit	EC$_{50}$ (μM ±SEM)	EC$_{50}$ mutant/EC$_{50}$ WT	-I_{max} (μA ±SEM)	n_H ±SEM
WT (5)	32.8 ± 2.5$^{\&}$	1.0	1.2 ± 0.1	1.33 ± 0.11$^{\&}$
S275T (5)	39.0 ± 2.0	1.2	1.6 ± 0.6	1.00 ± 0.20
L276A (6)	195 ± 27*	5.9	0.20 ± 0.01	1.27 ± 0.17
P277A (5)	82 ± 20*	2.5	1.1 ± 0.3	1.33 ± 0.17
V279A (4)	79.4 ± 6.5*	2.4	0.5 ± 0.1	1.36 ± 0.18
A280S (4)	46.6 ± 7.4	1.4	0.8 ± 0.5	0.96 ± 0.13
Y281F (5)	24.5 ± 2.7	0.7	1.1 ± 0.2	1.24 ± 0.13

Table 4-3. A comparison of the potency and efficacy of benzodiazepine site ligands at wild-type and V279A receptors. Data are expressed as mean ± SEM of at least 3 independent experiments. Levels of significance were determined by unpaired t-test. ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). [a] Wild-type values are from Dunn *et al.* (1999). Values for flunitrazepam and Ro15-4513 are EC_{50} and IC_{50} values, respectively. [b] Values for Ro15-1788 are reported as K_I (determined using the equation $K_I = IC_{50}/(1 + (L/K_d))$) (Cheng and Prussof, 1973).

Ligand	Wild-type[a]		V279A	
	Potency (nM)	Efficacy (% control)	Potency (nM)	Efficacy (% control)
Flunitrazepam	6.1 ± 0.4	256 ± 18	122 ± 6.0***	375 ± 65
Ro15-4513	1.8 ± 2.7	94.0 ± 1.2	40.0 ± 8.4**	78 ± 3.0**
Ro15-1788[b]	4.4 ± 0.1	—	19.0 ± 3.7*	—

BIBLIOGRAPHY

AMIN, J. AND WEISS, D.S. (1993). GABA$_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature*, **366**, 565-569.

AMIN, J., BROOKS-KAYAL, A., AND WEISS, D.S. (1997). Two tyrosine residues on the α subunit are crucial for benzodiazepine binding and allosteric modulation of γ-aminobutyric acid$_A$ receptors. *Mol. Pharmacol.*, **51**, 833-841.

BARNARD, E.A., SKOLNICK, P., OLSEN, R.W., MÖHLER, H., SIEGHART, W., BIGGIO, G., BRAESTRUP, C., BATESON, A.N., AND LANGER, S.Z. (1998). International union of pharmacology. XV. Subtypes of γ-aminobutyric acid$_A$ receptors: classification on the basis on subunit structure and receptor function. *Pharmacol. Rev.*, **50**, 291-313.

BONNERT, T.P., MCKERNAN, R.M., FARRAR, S., LE BOURDELLÈS, B., HEAVENS, R.P., SMITH , D.W., HEWSON, L., RIGBY, M.R., SIRINATHSINGHJI, D.J.S., BROWN, N., WAFFORD, K.A., AND WHITING, P.J. (1999). θ, a novel γ-aminobutyric acid type A receptor subunit. *Proc.Natl. Acad. Sci. U.S.A.*, **96**, 9891-9896.

BUHR, A., SCHAERER, M.T., BAUR, R. AND SIGEL, E. (1997). Residues at positions 206 and 209 of the α1 subunit of γ-aminobutyric acid$_A$ receptors influence affinities for benzodiazepine binding site ligands. *Mol. Pharmacol.*, **52**, 676-682.

CAMPOS-CARO, A., SALA, S., BALLESTA, J.J., VICENTE-AGULLO, F., CRIADO, M. AND SALA, F. (1996). A single residue in the M2-M3 loop is a major determinant of coupling between binding and gating in neuronal nicotinic receptors. *Proc.Natl.Acad.Sci. U.S.A*, **93**, 6118-6123.

CHENG, Y.-C. AND PRUSSOF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099-3108.

COLQUHUON, D. (1998) Binding, gating, affinity and efficacy: the interpretation of structure-activity relationships for agonist and of the effects of mutating receptors. *Br. J. Pharmacol.*, **125**, 924-947.

DAVIES, M., MARTIN, I.L., BATESON, A.N., HADINGHAM, K.L., WHITING, P.J. AND DUNN, S.M.J. (1996). Identification of domains in human recombinant $GABA_A$ receptors that are photoaffinity labelled by [^{3}H]flunitrazepam and [^{3}H]Ro15-4513. *Neuropharmacology*, **35**, 1199-1208.

DAVIES, M. AND DUNN, S.M.J. (1998). Identification of a unique domain in bovine brain $GABA_A$ receptor that is photoaffinity labelled by [^{3}H]Ro15-4513. *Biochem. Biophys. Res. Comm.*, **246**, 650-653.

DUNCALFE, L.L., CARPENTER, M.R., SMILLIE, L.B., MARTIN, I.L. AND DUNN, S.M.J. (1996). The major site of photoaffinity labeling of the $GABA_A$ receptor by [^{3}H]flunitrazepam is histidine 102 of the α subunit. *J. Biol. Chem.*, **271**, 9209-9214.

DUNN, S.M.J., DAVIES, M., MUNTONI, A.L. AND LAMBERT, J.J. (1999). Mutagenesis of the rat α1 subunit of the γ-aminobutyric $acid_A$ receptor reveals the importance of residue 101 in determining the allosteric effects of benzodiazepine site ligands. *Mol. Pharmacol.*, **56**, 768-774.

FISHER, J.L. AND MACDONALD R.L. (1998). The role of an α subtype M2-M3 His in regulating inhibition of $GABA_A$ receptor current by zinc and other divalent cations. *J. Neurosci.*, **18**, 2944-53.

GALZI, J.-L. AND CHANGEUX, J.-P. (1995). Neuronal nicotinic receptors: molecular organization and regulations. *Neuropharmacology*, **34**, 563-582.

KUME, A AND ALBIN, R.L. (1994). Quantitative autoradiography of 4'ethynyl-4-*n*-[2,3-$^3H_2$]propylbicycloorthobenzoate binding to the GABA$_A$ receptor complex. *Eur. J. Pharmacol.* **263**, 163-173.

KUSAMA, T., WANG, J., SPIVAK, C.E. AND UHL, G.R. (1994). Mutagenesis of the GABA ρ1 receptor alters agonist affinity and channel gating. *NeuroReport*, **5**, 1209-1212.

LANGOSCH, D., LAUBE, B., RUNDSTROM, N., SCHMEIDEN, V., BORMANN, J., AND BETZ H. (1994). Decreased agonist affinity and chloride conductance of mutant glycine receptors associated with human hereditary hyperekplexia. *EMBO J.*, **13**, 4223-4228.

MIHIC, S.J., WHITING, P.J., KLEIN, R.L., WAFFORD, K.A., AND HARRIS, R.A. (1994). A single amino acid of the human γ-aminobutyric acid type A receptor γ_2 subunit determines benzodiazepine efficacy. *J. Biol. Chem.*, **269**, 32768-32773.

MIHIC, S.J., YE, Q., WICK, M.J., KOLTCHINE, V.V., KRASOWSKI, M.D., FINN, S.E., MASCIA, M.P., VALENZUALA, C.F., HANSON, K.K., GREENBLATT, E.P., HARRIS, R.A., AND HARRISON N.L. (1997). Sites of alcohol and volatile anaesthetic action of GABA$_A$ and glycine receptors. *Nature*, **389,** 385-389.

NEWELL, J.G., DAVIES, M., BATESON, A.N., AND DUNN, S.M.J. (2000). Tyrosine of the γ-aminobutyric acid type A receptor β2 subunit is an important determinant of high affinity agonist binding. *J. Biol. Chem.*, **275**, 14198-14204.

PRITCHETT D.B. AND SEEBURG P.H. (1991). γ-Aminobutyric acid type A receptor point mutation increases the affinity of compounds for the benzodiazepine site. *Proc. Natl. Acad. Sci., U.S.A.*, **88**, 1421-1425.

RAJENDRA, S., LYNCH, J.W., PIERCE, K.D., FRENCH, C.R., BARRY, P.H. AND SCHOFIELD, P.R. (1994). Startle disease mutation reduces the agonist sensitivity of the human inhibitory glycine receptor. *J. Biol. Chem.*, **269**, 18739-18742.

RENARD, S., OLIVIER, A., GRANGER, P., AVENET, P., GRAHAM, D., SEVRIN, M., GEORGE, P. AND BESNARD, F. (1999). Structural elements of the γ-aminobutyric acid type A receptor conferring subtype selectivity for benzodiazepine site ligands. *J. Biol. Chem.*, **274**, 13370-13374.

SIGEL, E. AND BUHR, A. (1997). The benzodiazepine binding site of $GABA_A$ receptors. *Trends Pharmacol. Sci.*, **18**, 425-429.

SIGEL, E., BAUR, R., KELLENBERGER, S., AND MALHERBE, P. (1992). Point mutations affecting antagonist affinity and agonist dependent gating of $GABA_A$ receptor channels. *EMBO J.*, **11**, 2017-2023.

SIGEL E., BUHR, A. AND BAUR, R. (1999). Role of the conserved lysine residue in the middle of the predicted extracellular loop between M2 and M3 in the $GABA_A$ receptor. *J. Neurochem.*, **73**, 1758-1763.

UNWIN, N. (1993). Nicotinic acetylcholine receptor at 9Å resolution. *J. Mol. Biol.*, **229**, 1101-1124.

WIELAND, H.A., LÜDDENS, H. AND SEEBURG, P.H. (1992). A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J. Biol. Chem.*, **267**, 1426-1429.

CHAPTER 5[1]

Characterization Of The Interaction Of Zopiclone With γ-Aminobutyric Acid Type$_A$ Receptors

---

[1] *A version of this chapter has been accepted for publication.* DAVIES, M., NEWELL, J.G., DERRY, J.M.C., MARTIN, I.L. AND DUNN, S.M.J. (2000). *Mol. Pharmacol.*, **54.** Dr. Martin Davies and Jason Derry carried out the radioligand binding experiments. Dr. Ian L. Martin conducted the photoshift assay. Drs. Martin and Davies proposed the model for the benzodiazepine recognition site.

INTRODUCTION

$GABA_A$ receptors are heteromeric neurotransmitter receptors that belong to a ligand-gated ion channel superfamily that also includes the nicotinic acetylcholine, glycine, and $5HT_3$ receptors. Many different subunits for this receptor have been cloned, including α1-6, β1-3, γ1-3, ρ1–3, δ, π, ε and θ (for reviews, *see* Barnard *et al.*, 1998; Whiting *et al.*, 1999), and the pharmacological properties of any one receptor may be attributed to its particular combination of subunit isoforms. $GABA_A$ receptors are the site of action of many clinically important compounds, including the benzodiazepines, which interact with a specific binding site likely residing at the interface between the α and γ subunits (Davies *et al.*, 1996; Sigel and Buhr, 1997). Occupation of this site can produce a full spectrum of allosteric effects ranging from positive to negative modulation of GABA-gated ion flux (for reviews, *see* Sieghart, 1995; Barnard *et al.*, 1998).

When used clinically, benzodiazepines display anticonvulsant, sedative/hypnotic, myorelaxant and anxiolytic properties that can be exploited to ameliorate a variety of medical conditions. However, while highly efficacious and relatively safe, benzodiazepines are not suitable for chronic use because of the potential for development of tolerance. Other compounds that do not have a benzodiazepine structure but which also bind to this site display, in some cases, fewer of the side effects associated with classical benzodiazepines (Sanger *et al.*, 1994; Wagner *et al.*, 1998). Non-benzodiazepines such as the imidazopyridine zolpidem and the cyclopyrrolone zopiclone appear to be as efficacious as traditional benzodiazepines and are in clinical use (Goa and Heel, 1986; Wagner *et al.*, 1998). Zolpidem exhibits a lower potential for tolerance and abuse than the classical benzodiazepines and this has been attributed to its specific recognition of benzodiazepine type 1 receptors (Benavides *et al.*, 1988; Sanger *et al.*, 1994; Kunovac and Stahl, 1995). Zopiclone also appears to produce fewer unwanted side effects than the benzodiazepines (Julou *et al.*, 1985), but it is unknown if this is due to its preferential recognition of a particular receptor subtype.

While there is good evidence that the effects of zopiclone are mediated through GABA$_A$ receptors, the mechanism by which this occurs remains unclear (for review, *see* Doble *et al.*, 1995), although there is evidence to suggest that zopiclone allosterically modulates GABA$_A$ receptors in a unique fashion. The allosteric effects of zopiclone and flunitrazepam on [^{35}S]-*t*-butylbicyclophosphorothionate ([^{35}S]TBPS) binding appear to differ, in that the flunitrazepam- but not zopiclone-induced potentiation of [^{35}S]TBPS binding is antagonized by Ro15-1788 in a competitive manner (Lloyd *et al.*, 1990). *In vivo* binding studies have shown that the intra-peritoneal injection of zolpidem can decrease [^{3}H]Ro15-1788 binding in a dose-dependent manner in various mouse brain regions whereas the injection of zopiclone leads to increased binding of [^{3}H]Ro15-1788 (Byrnes *et al.*, 1992) but this may be a function of differences in pharmacokinetics. Furthermore, studies using rat brain membranes showed that, unlike [^{3}H]flunitrazepam binding, [^{3}H]zopiclone binding is not enhanced by secobarbital, pentobarbital or GABA (Blanchard *et al.*, 1983). In addition, there is no clear consensus on the efficacy of zopiclone at GABA$_A$ receptors. Functional studies have shown either no effect on (Concas *et al.*, 1994), a weak potentiation of (Skerritt and MacDonald, 1984), or a robust potentiation of (Reynolds and Maitra, 1996) GABA-gated chloride conductance.

Some groups have suggested that zopiclone produces fewer clinical side-effects than the classical benzodiazepines because it recognizes a unique binding site on the GABA$_A$ receptor. The evidence supporting the existence of this site is somewhat controversial. Data from kinetic studies suggest that the two sites are allosterically linked, as zopiclone was shown to accelerate the dissociation of [^{3}H]Ro15-1788 (Trifiletti and Snyder, 1984), although later studies were unable to repeat this finding (Concas *et al.*, 1994). In addition, equilibrium binding studies have suggested that the interaction of zopiclone and Ro15-1788 is non-competitive (Trifeletti and Snyder, 1984) while others have shown it to be competitive (Concas *et al.*, 1994). These discrepancies may be due to receptor heterogeneity in the variety of tissues and cell types used in these studies.

In photoaffinity labeling experiments, it has been shown that H101 of the $\alpha 1$ subunit is a major site of photoincorporation of [^{3}H]flunitrazepam (Duncalfe *et al.*, 1996) and this residue is an important determinant of benzodiazepine affinity (Wieland *et al.*, 1992; Davies *et al.*, 1998) and efficacy (Dunn *et al.*, 1999). Here we show that while photoaffinity labeling of the receptor markedly compromises the affinity of the classical benzodiazepines, zopiclone affinity is largely unaffected. However, we also show that mutating residue H101 results in similar changes in the binding profiles of both zopiclone and diazepam (see Fig. 5-1 for structures).

MATERIALS AND METHODS

Site-Directed Mutagenesis

Mutant α1 subunit cDNAs were generated as described previously (Davies *et al.*, 1998) using the Altered Sites® *in vitro* Mutagenesis System (Promega Corporation, Madison, WI, USA). Mutagenic oligonucleotides (described previously in Davies *et al.*, 1998) incorporated a silent restriction site that was used to initially screen for mutants. Those that were found to contain this site were then sequenced to confirm the presence of the desired substitution(s). The mutated cDNA was then subcloned into the expression vector pcDNA3.1(+) (Invitrogen).

Cell Culture and Transient Transfection

tsA201 cells were transfected with 10 μg of each subunit in a 1:1:1 ratio (mutant or wild-type α1:β2:γ2 or γ3) as previously described (Davies *et al.*, 1998). After 48 hours, the cells were harvested in ice cold buffer (Tris-HCl, pH 7.5) containing protease inhibitors. The cells were homogenized with two 10 second pulses using an Ultra Turrax homogenizer (IKA Labortechnik). Homogenates were washed by centrifugation and finally resuspended in ice-cold Tris-HCl buffer (pH 7.5). The homogenates were stored at -80°C until the day of the experiments.

Radioligand Binding Assays

Radioligand binding was performed in duplicate using a cell harvester (Brandel). For [^{3}H]Ro15-4513 saturation experiments, concentrations of radioligand ranging from 1 to 50 nM were used, with non-specific binding determined in the presence of 10 μM Ro15-4513. Cell homogenates were incubated with radioligand (and with a non-radiolabelled compound in the case of competition experiments) in 4°C buffer (50 mM Tris-HCl, 250 mM KCl, pH 7.4) for 1 hour prior to filtration. Following filtration, the filters were washed twice with 5 ml of ice-cold buffer. The filters were allowed to dry and were then placed in 5 ml scintillation vials. After the addition of 5 ml of scintillation fluid, the samples were counted for radioactivity.

Photoshift Experiments

For photoshift experiments, membranes were prepared from rat cerebellum. The tissue was placed in ice-cold 50 mM Tris-HCl buffer (pH 7.4) and homogenized for 10 seconds using an Ultra-Turrax homogenizer (IKA-Labortechnik). The homogenate was then centrifuged for 20 min at 40,000g at 4°C. Subsequently, the pellet was resuspended in the same volume of fresh buffer and centrifuged again. This step was repeated twice more for a total of three washes. After the final centrifugation, the pellet was resuspended in 20 volumes of buffer, frozen and stored at -80°C.

To perform photoshift experiments, membranes were thawed and diluted 1:80 to give a final protein concentration of approximately 1 mg/ml. The photoshift experiments were performed essentially as described by McKernan *et al.* (1998). The cerebellar homogenate was incubated for 30 min at 4°C in the presence of flunitrazepam, after which the mixture was exposed to ultraviolet light for 60 min. The membranes were then washed by centrifugation and resuspension six times to remove free flunitrazepam. To control for any changes to ligand recognition that might result from exposure of the receptor to UV light, membranes were irradiated in a similar fashion but in the absence of flunitrazepam during the 60-minute exposure period.

Homologous competition was used to determine the K_D value for [^{3}H]Ro15-1788. Displacement experiments were performed with 5 different concentrations of flunitrazepam and zopiclone. Non-specific binding was defined with clonazepam at a concentration of 3 μM. There was no difference between the non-specific binding in the photolabeled and the irradiated samples.

Electrophysiology

Oocytes from *Xenopus laevis* were maintained at 14 °C in Barth's solution (in mM):

NaCl (88), KCl, (1), $CaCl_2$(0.5), $Ca(NO_3)_2$ (0.5), $MgSO_4$(1), $NaHCO_3$ (2.4), 4-(2-(hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (15), pH 7.4. Oocytes were injected with 50 ng of cRNA for α1, β2 and γ2 or γ3 subunits in a ratio of 1:1:1. Approximately 48 hours later, the oocytes were used in concentration-response experiments. During the experiments, the oocytes were continuously perfused with frog Ringer's solution (in mM) NaCl (120), HEPES (5), KCl (2), $CaCl_2$ (1.8), pH 7.4. The eggs were impaled with two electrodes (resistances 0.5 - 2.0 ΩM in frog Ringer's solution) filled with 3 M KCl, and voltage clamped at –60 mV using a GeneClamp 500 amplifier (Axon Instruments Inc.). To examine potentiation of GABA-gated ion flux, the oocytes were pre-perfused with zopiclone or flunitrazepam for 3 min prior to the addition of GABA and the allosteric modulator. Potentiation experiments with $\alpha1\beta2\gamma2$ receptors were performed at the EC_{10} for GABA which was 5 μM. For γ3-containing receptors, potentiation was studied using the EC_{15} value (5 μM).

Data and Statistical Analyses

Ligand binding and electrophysiological data were analyzed using the curve fitting programs of GraphPad Prism (SanDiego, USA). Concentration-response curves for GABA-gated currents were fitted using the equation:

$$I = \frac{I_{max}[L]^n}{EC_{50}{}^n + [L]^n}$$

where I is the measured amplitude of the evoked current, $[L]$ is the concentration of GABA, $EC50$ is the GABA concentration that produces 50% of the maximal response (I_{max}) and n is the Hill coefficient. The modulation of the GABA response brought about by allosteric modulators was analyzed using the equation

$$I = I_o + \frac{(E_{max} - I_o)10^{Xn}}{10^{Xn} + 10^{Cn}}$$

where I is the amplitude of the observed current, X is the logarithm of the concentration of the allosteric modulator, I_0 is the current observed in the absence of modulator, E_{max} is the current observed at the maximally effective concentration, C is the logarithm of the EC_{50} for the response of the allosteric modulator and n is the Hill coefficient.

RESULTS

The Effects of Photolabeling on the Recognition of Flunitrazepam and Zopiclone

Flunitrazepam was used to covalently photolabel the receptor to examine the consequences on the subsequent binding of flunitrazepam and zopiclone. It has been previously shown that photoaffinity labeling with flunitrazepam causes a dramatic reduction in the affinity of the labelled receptor for classical benzodiazepines (Karobath and Supavilai, 1982; Thomas and Tallman, 1983; Brown and Martin, 1984). In our studies, photoaffinity labeling of rat cerebellar membranes reduced the affinity for flunitrazepam by approximately 50-fold (Table 5-1). However, the affinity for zopiclone was reduced only 3-fold (Table 5-1). The large decrease in flunitrazepam affinity with photolabeled cerebellar receptors mirrors the results obtained by others using recombinant human α1β3γ2 receptors (McKernan *et al.*, 1998).

Effects on Zopiclone and Diazepam Binding of Substitutions at Histidine 101 of the α1 Subunit

All ligand binding studies were carried out by competition with [^{3}H]Ro15-4513. As reported previously (Davies *et al.*, 1998) all mutant receptors examined retained high affinity for this ligand indicating that none of the mutations compromised the overall structure or expression of the receptors.

Wild-type α1β2γ2 receptors recognized zopiclone with an affinity of 51.3 ± 4.4 nM (Fig. 5-2 and Table 5-2), a value that closely matches previous reports with recombinant (Faure-Halley *et al.*, 1993) and native (Julou *et al.*, 1985) receptors. All of the mutations introduced at position 101 of the α1 subunit produced dramatic effects on the recognition of zopiclone when co-expressed with β2 and γ2 subunits, except for the phenylalanine mutant which produced a relatively modest 4-fold decrease in affinity (Fig. 5-2). The H101Q and H101Y mutations resulted in receptors having a 15- to 17- fold lower affinity than wild type receptors, whereas the H101A, H101K and H101E mutations produced receptors that did not recognize zopiclone in the concentration range used in these experiments (up to 10 μM).

The effects of these mutations on diazepam recognition paralleled those of zopiclone. Diazepam showed decreased recognition for all of the mutants in this study and completely failed to recognize H101A, H101K and H101E (Fig. 5-3 and Table 5-2). Again, as with zopiclone binding, the smallest shift in affinity for diazepam was observed with the phenylalanine substitution, which produced a 8-fold decrease in affinity.

Influence of γ Subunits on Zopiclone and Flunitrazepam Recognition

The identity of the γ subunit within the $GABA_A$ receptor oligomer can have profound effects on the pharmacology of ligands that interact with the benzodiazepine site (Tögel *et al.*, 1994, Lüddens *et al.*, 1994, Hadingham *et al.*, 1995). We therefore investigated the interaction of flunitrazepam and zopiclone with recombinant α1β2γ2 and α1β2γ3 receptors. Binding studies revealed that the K_D values for $[^3H]$Ro15-4513 binding were not significantly different between the two subtypes (Table 5-3 and Fig. 5-4; *see* Davies *et al.*, 1998 for comparison). However, the inclusion of the γ3 subunit in the receptor oligomer resulted in decreased affinity for both zopiclone and flunitrazepam, with a relatively greater decrease in flunitrazepam affinity compared to zopiclone (decreases of approximately 30- and 3-fold, respectively (Fig. 5-4). The concentration-effect relationship for GABA at α1β2γ3 receptors was established (Fig. 5-5) and, in good agreement with other published values, GABA was more potent (>2-fold) at α1β2γ3 receptors. The effects of flunitrazepam and zopiclone on GABA-gated currents mediated by α1β2γ2 and α1β2γ3 receptors were also examined (Fig. 5-6). Functionally, both zopiclone and diazepam were agonists at each receptor subtype, with flunitrazepam being the more potent of the two (Fig. 5-6 and Table 5-3). The substitution of γ2 with γ3 resulted in a rightward shift (approximately 3-fold) in the concentration-response curves for both agonists. Zopiclone produced a greater potentiation of GABA-gated current at both subtypes, having a larger effect at the γ3-containing receptors.

DISCUSSION

Zopiclone is an effective hypnotic agent that clearly produces its overt effects by interaction with the $GABA_A$ receptors in the central nervous system. Although the pharmacological spectrum of this agent is very similar to that of the classical benzodiazepines, there has been much speculation that the molecular mechanisms of action of the cyclopyrrolones and the benzodiazepines may be different.

Photoaffinity labeling of rat cerebellar membranes markedly compromised the affinity for flunitrazepam but had a much lesser effect on zopiclone binding (Table 5-1). These experiments were carried out using rat cerebellar membranes but the results are consistent with similar studies that used rat cortex membrane preparations (Blanchard *et al.*, 1983). The restricted expression of $GABA_A$ receptor subtypes in the rat cerebellum, compared to the cortex, suggest that the distinct consequences of photoaffinity labelling on the affinities of zopiclone and flunitrazepam are not due to the differential expression of $GABA_A$ receptor subtypes in these two brain regions.

Our previous studies showed that photoaffinity labeling with flunitrazepam results in covalent modification of H101 (Duncalfe *et al.*, 1996). We have, therefore, now compared the effects of point mutations of this residue on the binding characteristics of zopiclone and other benzodiazepine site ligands. The pattern that emerges from the binding studies strongly suggests that the residue at position 101 influences zopiclone binding in a similar manner to that of the classical benzodiazepines. As was previously observed with flunitrazepam and the β-carboline agonist ZK93423 (Davies *et al.*, 1998), aromatic residues and glutamine are generally well-tolerated substitutions for agonist recognition. The smallest of the amino-acid substitution, alanine, abolished recognition of both drugs, as did the glutamate and lysine substitutions. While interpretation of these mutagenesis studies requires more detailed knowledge of receptor-ligand interactions, the data show that the recognition properties of the mutants are similar for agonist ligands with disparate structures. This suggests that all agonists interact with H101 in a similar fashion and derive significant binding energy from this interaction.

McKernan *et al.* (1998) have recently investigated the effects of photoaffinity labeling with flunitrazepam on the binding of various benzodiazepine site ligands. They propose that ligands that display marked changes in affinity as a consequence of photoaffinity labeling derive binding energy through their interaction with H101, specifically through the pendant 5-phenyl substituent (C-ring) of the classical benzodiazepines. In contrast, they suggest that ligands that do not interact with H101 do not display reduced affinity for the photolabeled receptor. Previous modelling studies by Zhang *et al.* (1995) suggested that phenyl substituents in the R6 position of β-carboline agonists would occupy the same lipophilic pocket (L3) of the binding site as the C-ring of 1,4 benzodiazepine agonists (see Fig. 5-7). The results of the study by McKernan *et al.*, (1998) cast some doubt on the accuracy of this model, as they propose that, because photolabeling did not greatly reduce the affinity of abecarnil (a β-carboline with a phenyl substituent in the R6 position), this ligand does not interact with H101. Additionally, they suggest that ligands that do not show a large shift in affinity for photolabeled receptors (e.g. imidazopyridazines, cyclopyrrolones and β-carbolines) receive no binding energy from H101 and do not occupy this part of the pocket. The present results and those from previous studies indicate that this may not be the case and tend to support the models proposed by Zhang *et al.* (1995) as well as those of Fryer *et al.* (1986) and Gardner (1992). Previous mutagenic studies from this laboratory have shown that the β-carboline agonist ZK93423 displays a binding profile similar to flunitrazepam with several H101 mutants (Davies *et al.*, 1998). The results presented here show that diazepam and zopiclone also share this profile, suggesting that agonists at this site interact with H101 in a similar manner and derive binding energy from the interaction. However, zopiclone does not suffer a significant reduction in affinity as a consequence of receptor photolabeling, consistent with the notion that H101 does not form part of the L3 lipophilic pocket. These seemingly disparate findings can be explained by the fact that H101 is unlikely to be the only amino acid in the extracellular N-terminus that is involved in forming the recognition domain for these compounds. Our previous studies demonstrated that photolabeling with flunitrazepam occurred at several sites within the

$GABA_A$ receptor, only one of which was identified: H101 (Duncalfe *et al.*, 1996). Since the binding of these ligands to the receptor inevitably involves multiple points of interaction, it seems likely that the differential consequences of photoaffinity labelling on the affinities of flunitrazepam and zopiclone are a result of labeling at a site other than H101. The identification of this site must await further experimental studies.

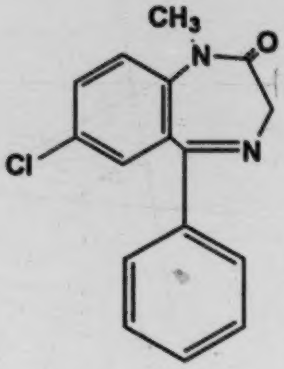
There is accumulating evidence to suggest that the benzodiazepine binding site is found at the interface between α and γ subunits (for review, *see* Sigel and Buhr, 1997). We have therefore also investigated the consequences of replacing the γ2 subunit with γ3 on the binding and function of zopiclone and the classical benzodiazepine flunitrazepam. In agreement with Reynolds and Maitra (1996), we find that zopiclone does potentiate GABA-gated current with recombinant receptors. Our data show that zopiclone is a full agonist at α1β2γ2 and α1β2γ3 receptors and is, in fact, more efficacious than flunitrazepam at both subtypes. Zopiclone produced a 32% greater potentiation than flunitrazepam at γ2-containing receptors and at γ3–containing receptors, the potentiation by zopiclone was 50% greater than that of flunitrazepam. Indeed, zopiclone appears to be a "super-modulator" at these receptors. Interestingly, while γ3-containing receptors showed a large reduction in affinity for flunitrazepam compared to γ2-containing receptors in radioligand binding assays using tsA201 cells (approximately 30-fold), the same degree of shift was not reflected in the electrophysiological data using *Xenopus* oocytes (2.5-fold). The reason for this discrepancy is unclear; generally, the ligand affinity values obtained from radioligand binding experiments tend to mirror closely the values obtained from electrophysiological experiments. However, we previously described a $GABA_A$ receptor mutant which displayed a binding constant that was 10-fold greater than the EC_{50} value obtained in functional studies (Davies *et al.*, 1998; Dunn *et al.*, 1999). The reason(s) underlying these instances in which binding and functional data seem not to match is currently unknown, although differences in expression systems such as post-translational modifications may play some role.

There has been considerable discussion about the molecular mechanism of zopiclone interaction with $GABA_A$ receptors; indeed here we show that the photolabeling of the receptor differentially affects zopiclone and flunitrazepam recognition. In the present study we demonstrate that zopiclone, like the classical benzodiazepines, interacts with the α1 subunit H101 residue of the benzodiazepine binding-site domain and further that the functional effects of these ligands are comparable. Thus the differences between zopiclone and the classical benzodiazepines that have been reported previously must be due to distinct interactions of these ligands with recognition site domains other than that represented by H101.

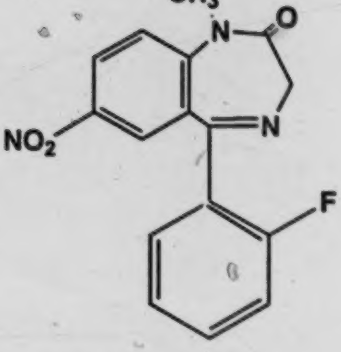
Figure 5-1

Chemical structures for (A) diazepam, (B) flunitrazepam and (C) zopiclone.

A.



B.


CH3
N
O
N
NO2
F

C.

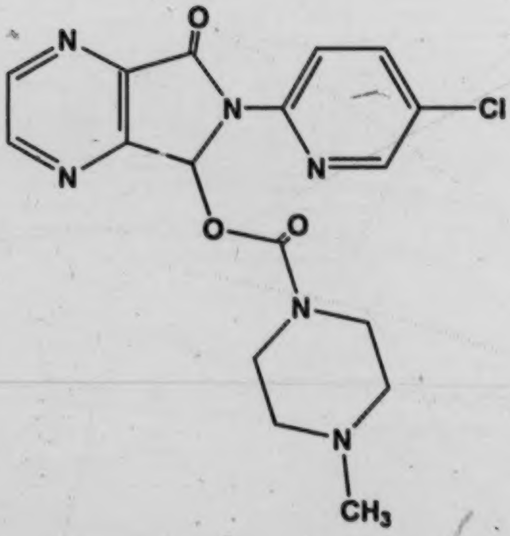

O
N
N
N
Cl
N
O
O
N
N
CH3

Figure 5-2

Competition curves showing the displacement of $[^3H]$Ro15-4513 by increasing concentrations of zopiclone from membranes prepared from cells expressing wild type (■), H101F (□), H101Q(●) and H101Y (○) α subunits coexpressed with $\beta2$ and $\gamma2$ subunits. $[^3H]$Ro15-4513 was present at a concentration equal to its K_D value for each receptor. Data shown represent the mean ± SEM of at least three independent experiments performed in duplicate.

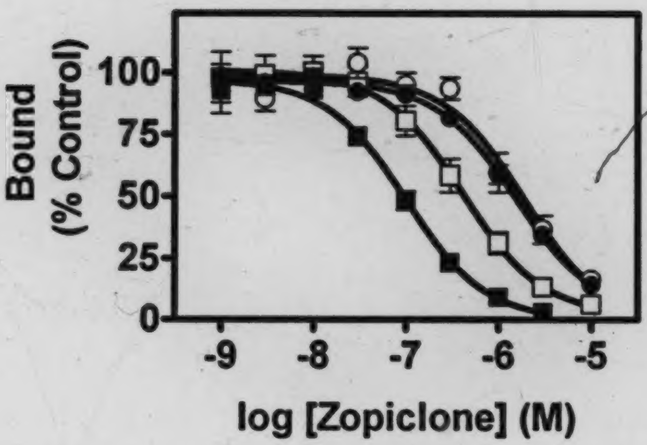

Bound
(% Control)
100
75
50
25
0
-9
-8
-7
-6
-5
log [Zopiclone] (M)

Figure 5-3

Competition curves showing the displacement of [^{3}H]Ro15-4513 by increasing concentrations of diazepam from membranes prepared from cells expressing wild type (■), H101F (□), H101Q(●) and H101Y (○) α subunits coexpressed with β2 and γ2 subunits. [^{3}H]Ro15-4513 was present at a concentration equal to its K_D value each receptor. Data shown represent the mean ± SEM of at least three independent experiments performed in duplicate.

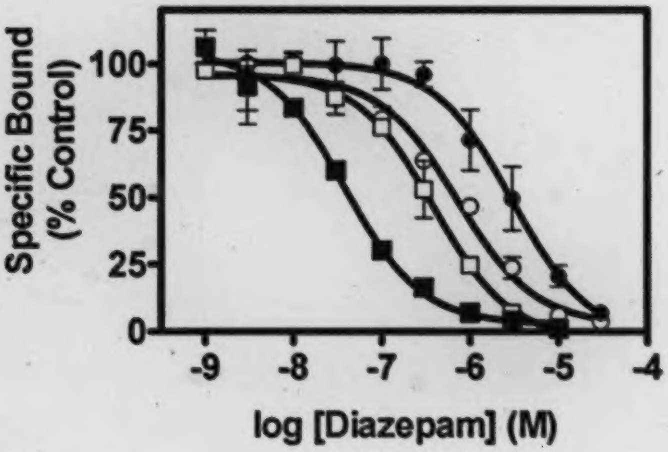

Specific Bound
(% Control)
100
75
50
25
0
-9
-8
-7
-6
-5
-4
log [Diazepam] (M)

Figure 5-4

The effect of the $\gamma 3$ subunits on the affinities of benzodiazepine site ligands. Inset, representative data showing the saturation analysis of [^{3}H]Ro15-4513 using membranes containing $\alpha 1\beta 2\gamma 3$ receptors. The experiment was repeated three times in duplicate. The K_D value was 10.4 ± 1.4 nM. The displacement of [^{3}H]Ro15-4513 by zopiclone (□) or flunitrazepam (■) from membranes containing $\alpha 1\beta 2\gamma 3$. The K_I values were 135 ± 16 and 202 ± 1 nM for zopiclone and flunitrazepam, respectively. Data shown represent the mean ± SEM for at least three independent experiments performed in duplicate.

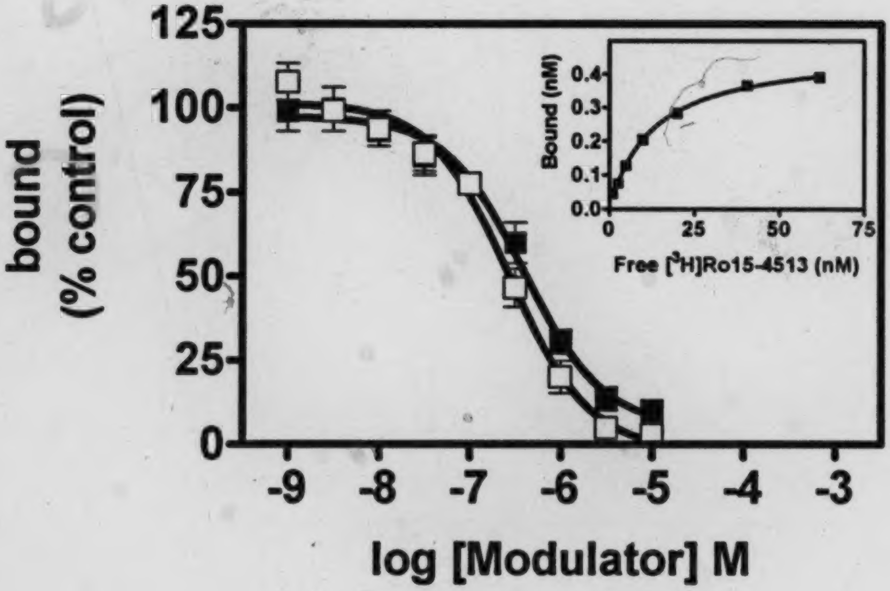

125
100
75
50
25
0
bound
(% control)
-9
-8
-7
-6
-5
-4
-3
log [Modulator] M
Bound (nM)
0.4
0.3
0.2
0.1
0.0
0
25
50
75
Free [³H]Ro15-4513 (nM)

Figure 5-5

(A). Concentration-dependence of agonist-activated Cl^- conductance for recombinant α1β2γ3 $GABA_AR$. Two-electrode voltage clamp analysis using *Xenopus* oocytes shows responses to GABA. The data represent the mean ± SEM of five independent experiments performed in duplicate. The EC_{50} for activation by GABA is 13.5 ± 3.7 μM. The Hill slope (n_H) for this curve is 1.12 ± 0.10. (B). Representative traces for concentration-dependence of agonist activated Cl- conductance for recombinant α1β2γ3 $GABA_AR$. The concentration applied at each interval is indicated (nM) above its respective trace.

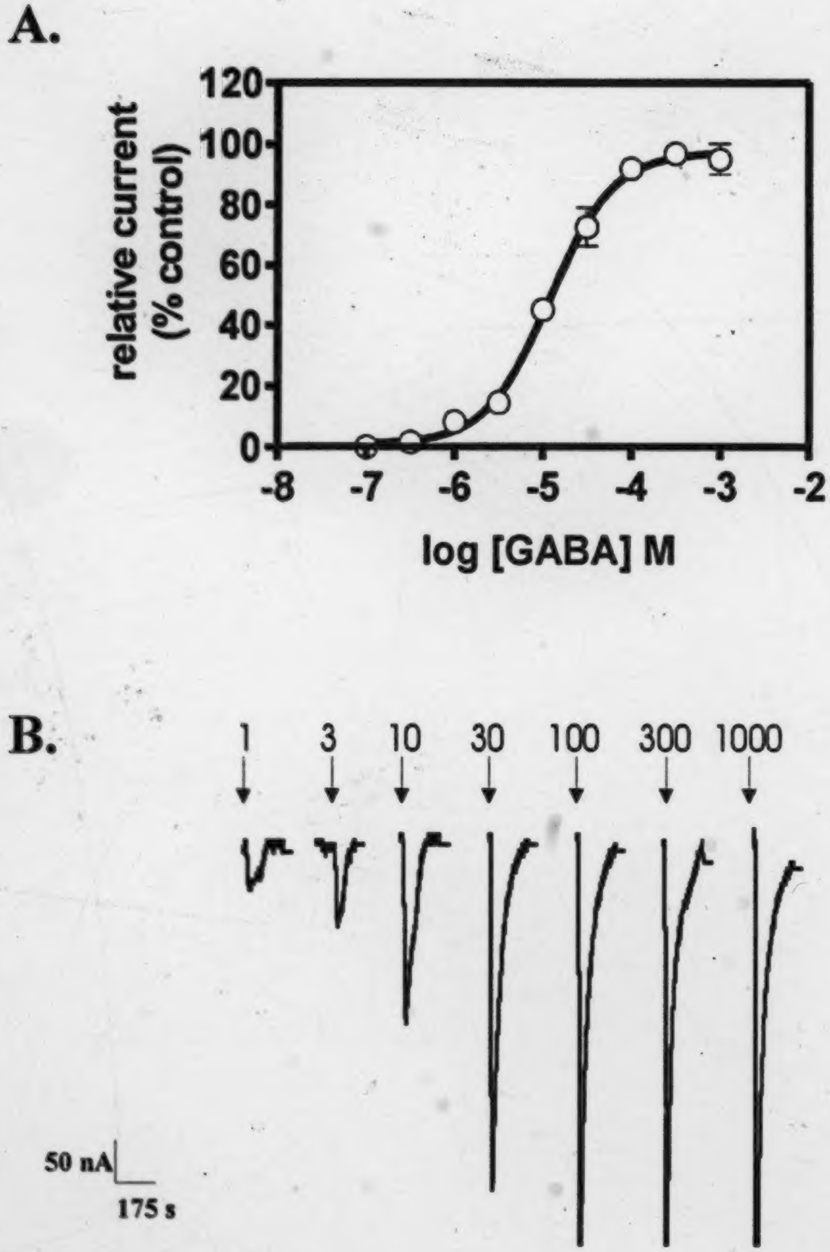
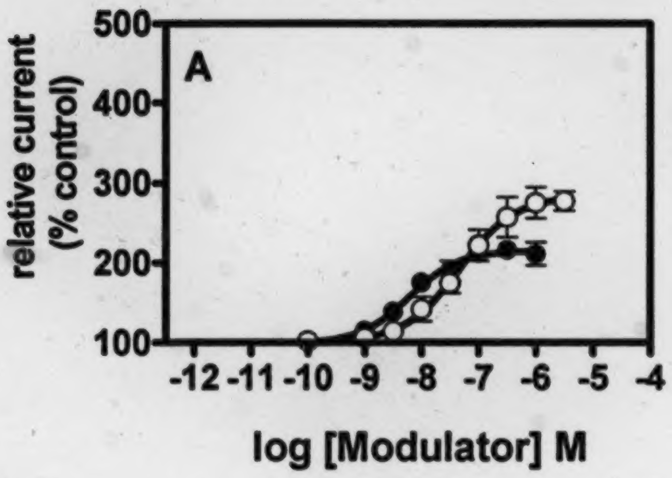

A.
120
100
80
60
40
20
0
relative current (% control)
-8
-7
-6
-5
-4
-3
-2
log [GABA] M
B.
1
3
10
30
100
300
1000
50 nA
175 s

Figure 5-6

Concentration-response curves illustrating the potentiation of GABA-mediated Cl^- conductance by flunitrazepam (●) and zopiclone (O) with recombinant $\alpha1\beta2\gamma2$ (A) and $\alpha1\beta2\gamma3$ (B) $GABA_AR$ expressed in *Xenopus* oocytes. Data represent the mean ± SEM of 3-4 independent experiments performed in duplicate. The EC_{50} values were $\alpha1\beta2\gamma2$ receptors were 5.9 ± 1.6 nM (●) and 42.4 ± 2.5 nM (O) for flunitrazepam and zopiclone, respectively. The potency of both modulators was shifted to the right in $\alpha1\beta2\gamma3$ receptors, and the EC_{50} values were increased to 14.8 ± 2.9 nM (●) and 135 ± 40 nM (O). E_{max} and log (EC_{50}) values were analysed by a two-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni t-test (Table 5-3) to determine the levels of significance.


A
relative current
(% control)
500
400
300
200
100
-12
-11
-10
-9
-8
-7
-6
-5
-4
log [Modulator] M

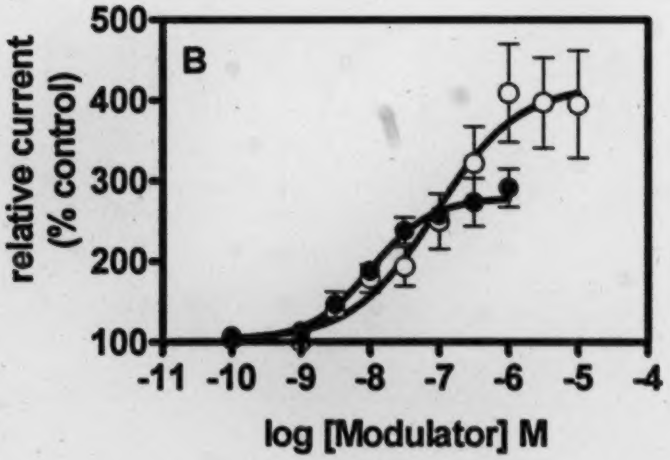
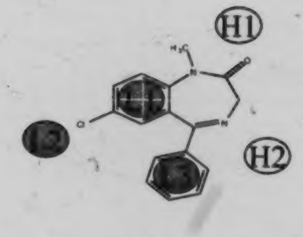
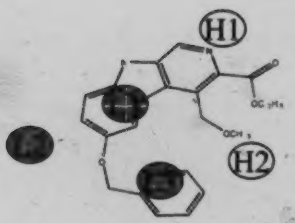

B
relative current
(% control)
500
400
300
200
100
-11
-10
-9
-8
-7
-6
-5
-4
log [Modulator] M

Figure 5-7

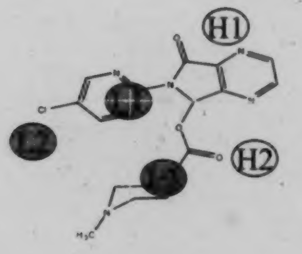
Orientation of diazepam, ZK93423 and zopiclone in the benzodiazepine pharmacophore of Zhang *et al.* (1995). The positions of the lipophilic pockets L1, L2 and L3, together with the hydrogen-bond donor sites H1 and H2 of the pharmacophore, have been used to orient both diazepam and ZK93423 as proposed in the agonist pharmacophore suggested by Zhang *et al.* (1995). The relative orientation of zopiclone is taken from that proposed by Borea *et al.* (1986).



diazepam



ZK93423


H1
H2
L2
N
O
Cl

zopiclone

● Lipophilic

○ H-bond donor

Table 5-1. Photolabeling-induced shifts in affinity for Ro15-1788, zopiclone and flunitrazepam. Values are in nM and are mean ± SEM, where n = 6 for Ro15-1788 and n = 3 for zopiclone and diazepam. Values are K_D for Ro15-1788 and K_I for zopiclone and flunitrazepam. (* $P < 0.01$ compared to irradiated samples).

Ligand	Control	Irradiated	Photolabeled	PAL/Irr. Ratio
Ro15-1788	2.03	0.97 ± 0.09	1.48 ± 0.22*	1.53
Zopiclone	29.5	29.6 ± 2.6	83.2 ± 6.7*	2.81
Flunitrazepam	6.8	4.6 ± 1.0	235 ± 39*	51.0

Table 5-2. K_I values (nM) for zopiclone and diazepam binding at $GABA_A$ receptors containing wild-type and mutant α subunits. Values are reported as mean ± SEM for at least three experiments performed in duplicate. [a] Values reported previously (Davies *et al.*, 1998).

α subunit	[^{3}H]Ro15-4513 (K_D)	Zopiclone (K_I)	K_I (mut)/ K_I (WT)	Diazepam (K_I)	K_I (mut)/ K_I (WT)
wild type	6.2 ± 0.54[a]	51.3 ± 5.4	1	19.4 ± 0.3	1
H101F	0.95 ± 0.07[a]	199 ± 28	4	164 ± 49	8
H101Y	7.01 ± 0.71[a]	863 ± 389	17	504 ± 51	25
H101Q	0.49 ± 0.07[a]	761 ± 216	15	1476 ± 782	74
H101E	2.13 ± 0.13[a]	> 10,000	-	> 10,000	-
H101K	1.54 ± 0.11[a]	> 10,000	-	> 10,000	-
H101A	8.3 ± 0.4	> 10,000	-	> 10,000	-

Table 5-3. Concentration-response data for flunitrazepam and zopiclone on GABA mediated current in recombinant $\alpha1\beta2\gamma2$ or $\alpha1\beta2\gamma3$ $GABA_AR$ expressed in *Xenopus* oocytes. Values (mean ± SEM) for the potency (EC_{50}), maximum potentiation (E_{max}) and Hill slope (n_H) were determined for each experiment (n) using GraphPad Prism Software. E_{max}, n_H and log (EC_{50}) values were analyzed using a two-way analysis of variance (ANOVA), followed by a *post-hoc* Bonferroni test to determine the levels of significance. Flunitrazepam was more potent at $\alpha1\beta2\gamma2$ and $\alpha1\beta2\gamma3$[a] receptors than zopiclone. Potency decreased at $\gamma3$-containing receptors for both flunitrazepam and zopiclone[b]. Zopiclone produced a greater maximal effect at $\gamma2$- and $\gamma3$[c]-containing receptors. Both compounds were more efficacious at $\gamma3$ containing receptors than at $\gamma2$ containing receptors[d].

Modulator	$\alpha1\beta2\gamma2$			$\alpha1\beta2\gamma3$		
	EC_{50} (nM)	E_{max} (%control)	n_H	EC_{50} (nM)	E_{max} (%control)	n_H
Flunitrazepam	5.9 ± 1.6 (3)	215 ± 4	1.00 ± 0.08	14.8 ± 2.9 (3)	280 ± 6	0.91 ± 0.26
Zopiclone	42.4 ± 2.5 (3)	284 ± 3	0.99 ± 0.06	135 ± 40 (4)	419 ± 25	0.72 ± 0.09

a. [F=58.68 (1,9), P<0.0001]
b. [F=15.04 (1,9), P<0.005]
c. [F=39.51 (1,9), P<0.001]
d. [F=36.53 (1,9), P<0.001]

BIBLIOGRAPHY

BARNARD, E.A., SKOLNICK, P., OLSEN, R.W., MÖHLER, H., SIEGHART, W., BIGGIO, G., BRAESTRUP, C., BATESON, A.N. AND LANGER, S.Z. (1998). International union of pharmacology. XV. Subtypes of γ-aminobutyric acid$_A$ receptors: classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.*, **50**, 291-313.

BENAVIDES, J., PENY, B., DUBOIS, A., PERRAULT, G., MOREL, E., ZIKVKOVIC, B. AND SCATTON, B. (1988). *In vivo* interaction of zolpidem with central benzodiazepine (BZD) binding sites (as labeled by [^{3}H]Ro15-1788) in the mouse brain. Preferential affinity of zolpidem for the ω_1 subtype. *J. Pharmacol. Exp. Ther.*, **245**, 1033-1041.

BLANCHARD, J.C., ZUNDEL, J.L. AND JULOU, L. (1983). Differences between the cyclopyrrolones (suriclone and zopiclone) and benzodiazepine binding to rat hippocampus photolabelled membranes. *Biochem. Pharmacol.*, **32**, 3651-3653.

BOREA, P.A., GILLI, G., BERTOLASI, V. AND FERRETTI, V. (1986). Stereochemical features controlling binding and intrinsic activity properties of benzodiazepine-receptor ligands. *Mol. Pharmacol.*, **31**, 334-344.

BROWN, C.L. AND MARTIN I.L. (1984). Photoaffinity labeling of the benzodazepine receptor compromises the recognition site but not its effector mechanism. *J. Neurochem.*, **43**, 272-273.

BYRNES, J.J., GREENBLATT, D.J. AND MILLER, L.G. (1992). Benzodiazepine receptor binding of nonbenzodiazepines in vivo; alpidem, zolpidem and zopiclone. *Brain Res. Bull.*, **29**, 905-908.

CONCAS, A., SERRA, M., SANTORO, G., MACIOCCO, E., CUCCHEDDU, T. AND BIGGIO, G. (1994). The effect of cyclopyrrolones on $GABA_A$ receptor function is different from that of benzodiazepines. *Naunyn Schmeid. Arch. Pharmacol.*, **350**, 294-300.

DAVIES, M., BATESON, A.N. AND DUNN, S.M.J. (1996). Molecular biology of the $GABA_A$ receptor: functional domains implicated by mutational analysis. *Frontiers Biosci.* (www.bioscience.org), **1**, d214-233.

DAVIES, M., BATESON, A.N. AND DUNN, S.M.J. (1998). Structural requirements for ligand interaction at the benzodiazepine recognition site of the $GABA_A$ receptor. *J. Neurochem.*, **70**, 2188-2194.

DOBLE, A., CANTON, T., MALGOURIS, C., STUTZMAN, J.M., PIOT, O., BARDONE, M.C., PAUCHET, C. AND BLANCHARD, J.C. (1995). The mechanism of action of zopiclone. *Eur. Psychiat.*, **10**, 117s-128s.

DUNCALFE, L.L., CARPENTER, M.R., SMILLIE, L.B., MARTIN, I.L. AND DUNN S.M.J. (1996). The major site of photoaffinity labeling of the $GABA_A$ receptor by [^{3}H]flunitrazepam is histidine 102 of the α subunit. *J. Biol. Chem.*, **271**, 9209-9214.

DUNN, S.M.J., DAVIES, M., MUNTONI, A.L. AND LAMBERT, J.J. (1999). Mutagenesis of the rat $\alpha 1$ subunit of the γ-aminobutyric $acid_A$ receptor reveals the importance of residue 101 in determining the allosteric effects of benzodiazepine site ligands. *Mol. Pharmacol.*, **56**, 768-774.

FAURE-HALLEY, C., GRAHAM, D., ARBILLA, S. AND LANGER, S.Z. (1993). Expression and properties of recombinant $\alpha_1\beta_2\gamma_2$ and $\alpha_5\beta_2\gamma_2$ forms of the rat $GABA_A$ receptor. *Eur. J. Pharmacol. (Mol. Pharmacol. Sect.)*, **246**, 283-287.

FRYER, R.I., COOK, Ch., GILMAN, N.W. AND WALSER, A. (1986). Conformational shifts at the benzodiazepine receptor related to the binding of agonists, antagonists, and inverse agonists. *Life Sci.*, **39**, 1947-1957.

GARDNER, C.R. (1992). A review of recently-developed ligands for neuronal benzodiazepine receptors and their pharmacological activities. *Prog. Neuro-Psychopharmacol. Biol. Psychiat.*, **16**, 755-781.

GOA, K.L. AND HEEL, R.C. (1986). Zopiclone: a review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy as an hypnotic. *Drugs*, **32**, 48-65.

HADINGHAM, K.L., WAFFORD, K.A., THOMPSON, S.A., PALMER, K.J. AND WHITING, P.J. (1995) Expression and pharmacology of human $GABA_A$ receptor containing γ3 subunits. *Eur. J. Pharmacol. (Mol. Pharmacol. Sect.)*, **291**, 301-309.

JULOU, L., BLANCHARD, J.C. AND DREYFUS, J.F. (1985). Pharmacological and clinical studies of cyclopyrrolones: zopiclone and suriclone. *Pharmacol. Biochem. Behav.*, **23**, 653-659.

KAROBATH , M. AND SUPAVILAI, P. (1982). Distinction of benzodiazepine agonists from antagonists by photoaffinity labeling of benzodiazepine receptors in vitro. *Neurosci. Lett.*, **31**, 65-69.

KUNOVAC, J.L. AND STAHL, S.M. (1995). Future directions in anxiolytic pharmacotherapy. *Psychiatr. Clin. North Am.*, **18**, 895-909.

LLOYD, G.K., DANIELOU, G. AND THURET, F. (1990). The activity of zolpidem and other hypnotics within the γ-aminobutyric acid ($GABA_A$) receptor supramolecular complex, as determined by ^{35}S-*t*-butylbicyclophosphorothionate (^{35}S-TBPS) binding to rat cerebral cortex membranes. *J. Pharmacol. Exp. Ther.*, **255**, 690-696.

LÜDDENS, H., SEEBURG, P.H. AND KORPI, E.R. (1994). Impact of β and γ variants on ligand-binding properties of γ–aminobutyric aid type A receptors. *Mol. Pharmacol.*, **45**, 810-814.

MCKERNAN, R.M., FARRAR, S., COLLINS, I., EMMS, F., ASUNI, A., QUIRK, K. AND BROUGHTON, H. (1998). Photoaffinity labeling of the benzodiazepine binding site of α1β3γ2 γ-aminobutyric $acid_A$ receptors with flunitrazepam identifies a subset of ligands that interact directly with his102 of the α subunit and predicts orientation of these within the benzodiazepine pharmacophore. *Mol. Pharmacol.*, **54**, 33-43.

REYNOLDS, J.N. AND MAITRA, R. (1996). Propofol and flurazepam act synergistically to potentiate $GABA_A$ receptor activation in human recombinant receptors. *Eur. J. Pharmacol.*, **314**, 151-156.

SANGER, D.J., BENAVIDES, J., PERRAULT, G., MOREL, E., COHEN, C., JOLY, D. AND ZIVKOVIC, B. (1994). Recent developments in the behavioural pharmacology of benzodiazepine (omega) receptors: evidence for the functional significance of receptor subtypes. *Neurosci. Biobehav. Rev*, **18**, 355-372.

SIEGHART, W. (1995). Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharmacol. Rev.*, **47**, 181-234.

SIGEL, E. AND BUHR, A. (1997). The benzodiazepine binding site of $GABA_A$ receptors. *Trends Pharmacol. Sci.*, **18**, 425-429.

SKERRITT, J.H. AND MACDONALD, R.L. (1984). Benzodazepine receptor ligand actions on GABA responses: benzodiazepines, CL 218872, zopiclone. *Eur. J. Pharmacol.*, **101**, 127-134.

THOMAS, J.W. AND TALLMAN, J.F. (1983). Photoaffinity labeling of benzodiazepine receptors causes altered agonist-antagonist interactions. *J. Neurosci.*, **3**, 433-440.

TÖGEL, M., MOSSIER, B., FUCHS, K. AND SIEGHART, W. (1994). γ-Aminobutyric acid$_A$ receptors displaying association of γ_3-subunits with $\beta_{2/3}$ and different α-subunits exhibit unique pharmacological properties. *J. Biol. Chem.*, **269**, 12993-12998.

TRIFILETTI , R.R. AND SNYDER, S.H. (1984). Anxiolytic cyclopyrrolones zopiclone and suriclone bind to a novel site linked allosterically to benzodiazepine receptors. *Mol. Pharmacol.*, **26**, 458-469.

WAGNER, J., WAGNER, M.L. AND HENING, W.A. (1998). Beyond benzodiazepines: alternative pharmacologic agents for the treatment of insomnia. *Ann. Pharmacother.*, **32**, 680-691.

WIELAND, H.A., LÜDDENS, H. AND SEEBURG, P.H. (1992). A single histidine in GABA$_A$ receptors is essential for benzodiazepine agonist binding. *J. Biol. Chem.*, **267**, 1426-1429.

WHITING, P.J., BONNERT, T.P., MCKERNAN, R.M., FARRAR, S., LE BOURDELLES, B., HEAVENS, R.P., SMITH, D.W., HEWSON, L, RIGBY, M.R., SIRINATHSINGHJI, D.J., THOMPSON, S.A. AND WAFFORD, K.A. (1999). Molecular and functional diversity of the expanding GABA-A receptor gene family. *Ann.N.Y. Acad. Sci.*, **868,** 645-653.

ZHANG, W., KOEHLER, K.F., ZHANG, P. AND COOK J.M. (1995) Development of a comprehensive pharmacophore model for the benzodiazepine receptor. *Drug Design and Discovery*. Harwood Academic Publishers, Heidelberg, Germany, pp 192-248.

CHAPTER 6

General Discussion

GENERAL DISCUSSION

Mutagenesis studies of the $GABA_AR$ have revealed important structural information about ligand binding domains for agonists, antagonists, allosteric modulators, and ion-channel blockers. The advent of this technology has permitted amino acid substitutions in putative binding domains to identify candidate amino acid residues with which ligands interact, to evaluate the relative contributions of specific residues to ligand recognition, and to determine the nature of the receptor-ligand interactions. This tool has proved to be particularly useful in conjunction with biochemical methodologies such as photoaffinity labeling and peptide mapping, which have been traditionally used to localize ligand-binding domains. However, the major limitation of site-directed mutagenesis is the fact that it is not possible to state with certainty, without a crystal structure of the receptor with the ligand bound, if alterations in ligand binding properties of agonists reflect local perturbations in the recognition pocket (i.e. binding) or more distant allosteric effects (i.e. channel gating). Thus, studies of this nature must be interpreted with caution.

Although mutagenesis is a powerful tool, it would be of little use to the protein chemist without heterologous expression tools. For the studies described in this thesis, transient transfection of tsA201 cells provided the means to investigate the binding parameters of agonists, antagonists, and allosteric modulators at recombinant $GABA_AR$. This cell line, derived from the widely used human embryonic kidney (HEK-293) cell line, was generated by the insertion of the gene for the temperature-sensitive SV40 T-antigen mutant, *tsA1609* (DuBridge *et al*., 1987) and, as a consequence, it expresses the T-antigen constitutively (Heinzel *et al*., 1988). Therefore, plasmids carrying an SV40 promoter will undergo replication that exceeds that of their normal promoter when transiently transfected (DuBridge *et al*., 1987). While these cells provide abundant expression of protein for radioligand binding experiments, their use for functional studies is limited by low expression efficiency and the fact that it is difficult to determine which cells express the protein of interest. Therefore, the *Xenopus* oocyte expression system was selected for functional studies. With few exceptions, the information obtained from one system usually compares favorably with that obtained from the other. For example, the potency of allosteric modulation of GABA gated current by benzodiazepine ligands is in excellent agreement with the affinity determined from binding studies in tsA201 cells (*see* Dunn *et al.*, 1999 and Davies *et al*, 1998). However, there are examples in which the potency for potentiation of GABA current by flunitrazepam greatly exceeds that of the binding affinity as determined in tsA201 cells (see Chapter 5). These subtle discrepancies presumably reflect differences in receptor assembly processes and posttranslational modifications in each heterologous expression system (*see* Burt and Kamatachi, 1991). Nevertheless, it is possible to use both expression systems to address a specific question.

One of the problems associated with transient transfection is the lack of knowledge of receptor assembly in each batch of cells. Although many protocols exist for the transient transfection of any particular cell line, the calcium-phosphate method as outlined by Chen and Okayama (1987) provides an efficient and cost-effective method to introduce DNA into cells. It is generally accepted that a transfection ratio of equal amounts of the α, β and γ subunits in a 1:1:1 ratio (as carried out in the studies described in this thesis) produces a single population of receptors within a given cell. Although this ratio has been varied to 1:1:5-20 to ensure expression of γ subunits in each receptor (Sigel *et al.*, 1992; Boileau *et al*, 1998; Boileau *et al.*, 1999; Sigel *et al.*, 1999), it has been shown by single channel analysis that transfection of αβγ $GABA_AR$ subunits in HEK-293 cells in an equimolar ratio preferentially produces αβγ receptors (Angelotti *et al.*, 1993). However, this does not exclude the possibility that multiple populations are present within the cell (*see* Sieghart, 1995) since only single channel analysis can readily resolve minor populations of αβ receptors, which can be distinguished from their αβγ counterparts on the basis of their main and subconductance states (Angelotti and Macdonald, 1993). However, despite these elegant single channel data, there are indications that the transfection of multiple subunits can produce a heterogeneous population of receptors when introduced into HEK-293 cells. It has been specifically demonstrated that transfection of α4β3γ2 (in a 2:1:1 ratio) produces mixtures of α4β3γ2, α4β3, and β3 receptors within a population of cells (Ebert *et al.*, 1996). This was suggested on the basis that the ratio of muscimol:Ro15-4513 binding sites was >5:1 and that GABA was only able to displace 20% of the total TBPS binding, which is in marked contrast to the complete displacement of TBPS by GABA measured in α1β3γ2 receptors. However, this observation could reflect inefficient assembly of this $GABA_AR$ subtype.

Although the use of electrophysiological techniques likely precludes the measurement of subunit combinations that are preferentially retained within the ER, radioligand-binding studies carried out on homogenates can detect both cell surface and receptor intermediates. For the radioligand binding experiments described in the present dissertation, preliminary single point binding assays suggested that both Ro15-4513 and high affinity muscimol binding sites were present in a single batch of transfected cells (data not shown). Homogenates that did not meet this criterion were not used for radioligand binding experiments. Although these results could be indicative of intracellular αγ heterodimers and cell surface αβ receptors, we think this unlikely since GABA potentiated flunitrazepam binding in all recombinant receptors (30-75%) whereas the potentiation of flunitrazepam binding at αγ receptors is weak (<20%) (Ebert *et al.*, 1996). However, the presence of minor populations of either αβ or αγ receptors cannot be excluded since the affinity of muscimol for αβ and αβγ receptors in indistinguishable (Pregenzer *et al.*, 1993), as is the affinity for Ro15-4513 (6.3 nM) at αγ and αβγ receptors (Wong *et al.*, 1992). The only subtle distinction between αγ dimers and αβγ trimers is that αγ receptors have a 10-fold lower binding density for Ro15-4513 (Ebert *et al.*, 1996), whereas the binding density for muscimol in αβ and αβγ receptors is comparable (Ebert *et al.*, 1996).

The greatest difficulty with mutating receptors to investigate agonist-binding domains is the interpretation of the results in terms of their effects on ligand binding and or channel gating. Some attempts to define the "rules" by which such interpretations should be made have been put forth. In an attempt to define categorically the effects of mutations on agonist-binding domains of nAChRs, Galzi *et al.* (1996) proposed a phenotypic classification system for changes in ligand binding and channel gating properties, although it has long been recognized that the two are interdependent processes (Chang and Weiss, 1999). Therefore, mutations at any position in the primary sequence of a protein will often produce almost identical phenotypes, irrespective of the role of any given amino acid in receptor function. To that end, Galzi *et al.* (1996) proposed that mutations be classified according to the binding (κ), isomerization (L) and conductance (γ) phenotypes. Mutations that alter the intrinsic binding properties (i.e. those directly involved in the binding of agonist molecules) can manifest their effects in one of two ways. If it is assumed that a binding site has only two conformations and that there are not multiple independent binding sites on a single receptor, mutations can alter the affinity for both conformations (i.e. active and desensitized), but affinity ratio between the two will remain constant. In such cases, there will also be a concomitant rightward shift in the concentration-response curve, without alterations in the Hill slope or the maximum response. However, if mutations alter the affinity for only one of the two receptor states, then the affinity ratio changes and there will be a decrease in both the Hill slope and the maximum response (Galzi *et al.*, 1996). Isomerization phenotypes are assumed to arise from changes to the conformational machinery of the receptor that alter the equilibrium between two interconvertible states (with no changes in affinity or ion channel activity). Conductance phenotypes, on the other hand, alter the activity of the ion channel such that a non-conducting channel becomes a conducting one (e.g. in the absence of ligand). Therefore on the basis of these criteria, it may be possible to assign the κ phenotype to the mutations at position 62 of the mature β2 subunit on the basis of the data presented in Chapter 2 alone. Clearly, many of the criteria are satisfied such that there are changes in the affinity as determined using ligand binding techniques and in the concentration-dependence of the GABA/muscimol current as determined in two-electrode voltage clamp experiments but there is no significant change in Hill number. The Hill coefficient, however, does not define any physical mechanism and absolutely no conclusions can be made based on this number alone (Colquhoun, 1998). In fact, the Hill slope is only indicative of the minimum number of agonist molecules required to gate the channel but does not provide a precise number since the number of molecules in unquestionably greater than that indicated by the Hill coefficient. Further, this value can be high when multiple cooperative binding sites are present (i.e. the affinity of a second site is enhanced by occupancy of the first) (Colquhoun, 1998). Additionally, changes in the maximum response are difficult to interpret for mutant receptors since the efficiency of transfection can vary for each batch of cells and there is no guarantee that the mutant constructs will be as efficient as the wild-type constructs (Colquhoun, 1998). Moreover, the criteria established by Galzi *et al.* (1996) do not take into account multiple receptor binding sites at equilibrium and largely serve to explain the changes in ligand binding properties strictly on the basis of the Monod-Wyman-Changeux model of allosteric proteins alone.

Equally difficult to rationalize is the nature of the binding site on the basis of information obtained exclusively from equilibrium binding studies. Since agonists elicit conformational receptor changes, it is likely that amino acids other than those identified in equilibrium binding studies will contact the ligand; it is equally possible to speculate that those identified in radioligand binding studies may not even contact the neurotransmitter in the active state (Colquhoun, 1998). Concentration-response studies are often limited by the fact that the maximum response is susceptible to desensitization phenomena and that in itself, can influence the apparent affinity of the ligand in the active state. Thus, while preliminary information obtained on high affinity agonist binding and its functional role provides unique insight into $GABA_AR$ structure and function, these studies will be greatly enhanced by (1) single channel data to provide a more detailed analysis as to the efficacy and (2) biophysical experiments to measure agonist-induced receptor conformational changes. Recently, however, some resolution of this issue has been achieved with respect to $GABA_C$ receptors, which, unlike their $GABA_A$ counterparts, do not desensitize. The exploitation of this phenomenon allowed Chang and Weiss (1999) to use the *Xenopus* oocyte expression system to measure ligand binding and channel gating in the same cells; in this assay, it was shown that the concentration required for ion channel activation and half-maximal binding were almost identical.

One of the major goals of this work was to understand the functional relevance of the high affinity agonist binding site(s) of $GABA_ARs$. To date, it has generally been considered that the only agonist binding sites of relevance were the (low affinity) channel activation sites that have been well characterized in terms of their location on $GABA_AR$ (Sigel *et al.*, 1992; Amin and Weiss, 1993; Smith and Olsen, 1994; Boileau *et al.*, 1999; Westh-Hansen *et al.*, 1999). Although many investigators have noted the existence of high affinity binding sites (*see* Im *et al.*, 1997), there have been no attempts to identify the structural basis for high affinity agonist binding. This has occurred primarily for one reason. Many groups consider the high affinity agonist binding site to simply represent the desensitized state of the channel activation sites (*see* Sigel and Buhr, 1997)

The reasons for which a single receptor molecule would carry multiple agonist binding domains of different affinity are unclear. A theoretical modeling paper of an idealized nACnR (Jackson, 1989) suggests that binding energy can be utilized in two ways by ligand-gated ion channels, including stabilization of the closed conformation of the receptor and induction of the open state of the receptor from the resting or inactive state (Jackson, 1989). In order to satisfy the physiological requirement for rapid receptor activation, multiple non-equivalent binding sites are ideal. Occupation of high affinity binding sites may increase the affinity of low affinity channel activation sites to drive the receptor into the open conformation and, in mutated receptors, binding sites of similar or equivalent affinity require more energy to achieve the open state as do their wild-type counterparts. This serves to limit the amount of energy available for desensitization processes and could serve to explain the basis for the phenomenological finding that receptors in which Y62S (which lacks measurable high affinity binding) is expressed recover in the presence of agonist perfusion. Therefore the energetics of the systems are better defined when two non-equivalent binding sites are present. Multiple non-equivalent binding sites therefore permit the receptor to function optimally and permits rapid activation and termination of the response (Jackson, 1989).

CONCLUSIONS

Although the current data have identified Y62 of the β2 subunit as a residue that is important in conferring high affinity for muscimol and GABA, it is not possible, on the basis of these data alone, to construct a detailed model of how these ligands interact with the receptor. Further, it cannot be unequivocally assumed that this residue is important for the direct interaction of these molecules and not simply required for the maintenance of the physiochemical characteristics of the high affinity binding domain. Both GABA and muscimol are zwitterionic at physiological pH. Intuitively, it would be assumed that on adjacent subunits there could exist an anionic and cationic subsite that would stabilize the interaction with the positive and negatively charged moieties of these agonists, respectively. Molecular modeling of the GABA binding site has predicted that arginine and aspartate residues could, in fact, fulfill these roles, in addition to hydrogen bonding with threonine and tyrosine residues (Aprison *et al.*, 1992; Galvez-Ruano *et al.*, 1995;

Aprison *et al.*, 1996). Although these models do not include the full complement of residues from adjacent subunits that form part of the same binding pocket, they do provide some theoretical considerations that could be important in the identification of other domains and residues which play a role in ligand binding.

In the structurally homologous neurotransmitter binding domain of the nAChR, aromatic residues such as phenylalanine, tyrosine, and tryptophan have largely been implicated in the formation of a negative subsite which stabilize the interactions with the positively charged quaternary ammonium group of acetylcholine. This stabilization is reported to occur via cation-π stacking effect in which the uneven distribution of charge above and below the plane of the aromatic rings provides sufficient negative charge for the interaction of the amino acids with the ligand. Clearly, in the studies detailed in Chapter 2, the aromaticity of the tyrosine residue is important since it effectively rescues binding when compared to the serine substitution. However, because the affinity is not restored to wild-type levels (~6-fold lower), it is equally likely that the hydroxyl group plays an important role in the receptor-ligand interactions, but in the Y62S mutation may be physically distant from GABA/muscimol and therefore cannot interact with the agonist. Although other mutagenesis studies have shown that amino acid substitutions affect the affinities of GABA and muscimol differentially (Amin and Weiss, 1993), this need not be the case since muscimol is a conformationally restricted analogue of GABA and therefore shares many of the same physicochemical characteristics.

Similarly, it is difficult to explain in terms of a model, the interaction of benzodiazepine ligands with V279 of the α1 subunit. While amino acids within this same domain have been implicated in the formation of the zinc binding site (Fisher and Macdonald, 1998; Hornstein and Akabas, 1998), the neurosteroid binding site (Mihic *et al.*, 1997) and an alcohol binding site (Mihic *et al.*, 1997), there are no reports of mutations in this region affecting the pharmacology of the benzodiazepines. The finding that substitution at V279 shifts the affinity of flunitrazepam and Ro15-4513, while at the same time increasing the apparent efficacy of both compounds presents an interesting problem. Since the mutation itself had no major effect on the maximum current elicited by application of agonist, while at the same time shifted the agonist concentration-effect curve to the right suggests that this residue could be important for conferring affinity to agonists. It is tempting to speculate that this residue is somehow important for the binding of these ligands. This was not expected since the high affinity-binding site for these benzodiazepines is believed to be located at the interface between the amino-terminal domains of the α and γ subunits (see Introduction), which is approximately 3.0 nm from the extracellular loop (Unwin, 1995). However, it is interesting to note that the apparent affinity of flunitrazepam closely corresponds to a lower affinity component that has been noted in α1β2γ2 receptors (Dunn *et al.*, 1999). Therefore, the novel labeling of this domain by Ro15-4513 (Davies and Dunn, 1998) may indicate that there is a second site for benzodiazepine binding and this additional lower affinity site has been noted by other investigators (Im *et al.*, 1993). It will be important to establish the interactions between His-101 of the α1 subunit and this residue to see if they are, in fact, involved in the formation of a deep pocket with which the benzodiazepines interact or if this represents a novel benzodiazepine binding site on the receptor.

The studies on desensitization of $GABA_AR$ as presented in Chapter 3 have produced the first evidence that disruption of high affinity agonist binding affects the desensitization properties of the receptor. It is tempting to put the ideas of this work into a novel model to explain activation and desensitization of a receptor that carries two distinct populations of binding sites on a single receptor molecule. Classical models that suggest there are multiple states of a single binding site with different affinities fail to explain adequately these phenomena. Specifically, the model of Monod-Wyman-Changeux does not take into account the fact that there are multiple binding sites of different affinity but states that the affinity of single binding site changes and increases as the receptor passes from the active state to the intermediate state to the desensitized state. Further, since recovery of $GABA_AR$ from desensitization is presumed to be almost entirely dependent on dissociation of the ligand from its high affinity state, it is tempting to rework the model to include multiple binding sites. This would clearly be explained if an alternate model of nAChR activation is taken into account in which the binding to multiple sites is simple but channel activation requires cooperativity among all binding sites (Raftery *et al.*, 1983). Thus, in wild-type receptors, where the high affinity binding site is intact, there is a long 'wash-out' period required for functional recovery. However, when high affinity binding is abolished (as is the case with Y62S), recovery occurs during the presence of continuous agonist perfusion. Therefore, although low affinity binding sites mediate channel activation, their continuous occupation is not enough to prevent recovery from desensitization, presumably from a receptor conformational change. This therefore suggests that multiple agonist binding sites of different affinity fulfill specific functional roles.

While most of the projects have involved examination of the interaction of agonists with their receptor, allosteric compounds have also been studied and this has provided a broader perspective on GABA$_A$R function. The interaction of zopiclone has also been examined in greater detail to gain a better perspective of the interaction of this compound with GABA$_A$R. Since this compound was designed to be a sedative-hypnotic and to interact much in the same way that zolpidem and other classical benzodiazepine agonists do, it is unclear as to why some authors would suggest that this compound does not derive significant energy from interaction with His 101. On the basis of radioligand binding, photolabeling, and electrophysiology, it is clear that zopiclone interacts in much the same manner, as does diazepam with the exception of its interaction with the γ3 subunit.

FUTURE DIRECTIONS

There are multiple approaches that will enable further investigation of the high affinity agonist binding domain. The use of alanine-scanning mutagenesis or the substituted cysteine accessibility method will undoubtedly lead to the identification of additional residues within loop D of the β2 subunit that contribute to the high affinity agonist binding domain. Alanine-scanning mutagenesis is reported to be a more powerful approach than homologue mutagenesis (Cunningham and Wells, 1988) because alanine substitutions maintain the integrity of the backbone of the polypeptide chain, while, at the same time, eliminate side chain interactions (Ward *et al.*, 1990). Alternatively, introduction of cysteine residues in this binding domain, followed by modification of these residues by MTS derivatives, would provide more information as to the accessibility of specific residues to muscimol for either ligand binding or channel activation. This approach provides information about a binding site because it identifies residues with which the ligands make contact (*see* Colquhoun, 1998). Both alanine-scanning mutagenesis and SCAM technology will provide additional information as to the secondary structure of this loop, which, in the homologous $5HT_{3A}$ receptor and α1 subunit of the $GABA_AR$ is reported to be a β strand (Yan *et al.*, 1999; Boileau and Czajkowski, 1999). Another approach to localize the high affinity binding site will be future photolabeling studies with [^{3}H]muscimol or the recently synthesized [^{3}H]thiomuscimol, which is reported to incorporate more efficiently into the protein. This approach, combined with peptide mapping and protein sequencing, will provide important biochemical confirmation of the localization of the label, which, when combined with mutagenesis data, will provide powerful information as to the location of the high affinity binding site. The advance of [^{3}H]muscimol photolabeling will represent a significant improvement in the investigation of binding sites on the $GABA_AR$ because previous attempts to sequence proteins subsequent to photolabeling have provided limited information (Deng *et al.*, 1986; Casalotti *et al.*, 1986; Bureau and Olsen, 1988; Smith and Olsen, 1994).

It will also be important to establish the structural requirements for ligand binding at Y62 and at other positions identified using the approaches described above in accordance with the study of Davies *et al.* (1998), in which multiple amino acid substitutions at a single position facilitated the determination of structural determinants for benzodiazepine ligand binding. Further, identification of amino acid residues of the α1 subunit that contribute to the high affinity agonist binding domain will further allow for the resolution of the binding site. This may be accomplished by the generation of α/γ chimeras, followed by expression with β2 and γ2 subunits. Changes in the apparent affinity by mutations at position 101 of the α1 subunit produced comparable changes to those observed by Newell *et al.* (2000a) and Dunn *et al.* (1999), which suggests that high affinity binding site determinants at the α–β interface may include determinants of the high affinity benzodiazepine site. The reason for this potential overlap is that the loops of the α subunit (A-C) which complement loop D of the β2 subunit for the formation of a high affinity site, include the same loops as those in which residues have been identified as being important for the binding of benzodiazepine ligands.

With respect to the desensitization of $GABA_AR$, the finding that loss of high affinity binding permits recovery of $GABA_AR$ during continuous agonist perfusion requires the use of patch-clamp technology to reliably quantitate these phenomena. The use of patch-clamp technology with these mutants will allow for the determination of kinetic constants such as on and off rates that will undoubtedly enhance the data from the *Xenopus* oocyte expression system. Because the superfamily of ligand gated ion channels shares sequence identity and appear to share many common mechanisms for receptor activation and desensitization, it will be important to study other receptors to determine if the protocols established for studying desensitization phenomena of $GABA_AR$ with the *Xenopus* expression system provide similar information on the desensitization processes of the $5HT_3R$, the nAChR, and the GlyR. The use of this system may provide for a more reliable quantitation of the apparent affinity of the endogenous neurotransmitters for each of these receptors at their low affinity binding sites. The identification of high affinity binding site determinants in other receptors and investigation of the functional roles of these sites will allow for the determination of general mechanisms of desensitization for all ligand-gated ion-channels.

With respect to the benzodiazepine studies, it will be important to explore further the contributions of the loop between transmembrane domains II and III to ligand binding. The role of V279 could be demonstrated by making homologous mutations in the β and γ subunits; if the residue was important in binding, then the mutation in the β subunit should not affect the apparent affinity of flunitrazepam or Ro-15-4513 since the benzodiazepine binding site is generally considered to be located at the interface of the α-γ subunits. It will be important to make multiple substitutions at this position, again to identify structural requirements for benzodiazepine-site ligands. It will also be interesting to see if it is possible to cross-link V279 with H101 and determine if the two residues clearly contribute to the same binding site via protein-protein interactions.

More global directions include the crystallization of the $GABA_AR$, a prospect that has thus far eluded the scientific community because (1) of the limited amount of protein from any given source and because (2) the lipid environment of the cell membrane makes it difficult to determine the crystal structures of membrane-bound proteins. Until that point, it is impossible to know if the point mutations that have been introduced into the receptor alter the binding or gating properties, though most researchers have made strong arguments in favour of one or the other on the basis of their data. Although structural data in terms of the secondary structure of the receptor are now emerging, it will also be important to cross-link residues of adjacent loops to map the spatial arrangement of the loops and the distance between them and, indirectly, between adjacent subunits.

In conclusion, there are many structural aspects of the GABA$_A$R that will be important to explore in future years. Because this receptor possess a rich pharmacology, it will be necessary to elucidate structural requirements for agonist and modulator binding with the hopes of designing therapeutic agents which lack classical side effects of the benzodiazepines, such as respiratory depression, and short-term memory loss. Molecular and structural studies as to receptor regulation, synthesis, degradation and ligand binding domains will undoubtedly provide answers to important questions that will be beneficial to both the basic and medical sciences alike.

BIBLIOGRAPHY

AGEY, M.W. AND DUNN, S.M.J. (1989). Kinetics of [^{3}H]muscimol binding to the GABA$_A$ receptor in bovine brain membranes. *Biochemistry*, **28**, 4200-4208.

AMIN, J. AND WEISS, D.S. (1993). GABA$_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature*, **366**, 565-569.

AMIN, J., DICKERSON, I.M. AND WEISS, D.S. (1994). The agonist binding site of the γ-aminobutyric acid type A channel is not formed by the extracellular cysteine loop. *Mol. Pharmacol.*, **45**, 317-323.

ANGELOTTI, T., P. AND MACDONALD, R.L. (1993). Assembly of GABA$_A$ receptor subunits: $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2S}$ subunits produce unique ion channels with dissimilar single-channel properties. *J. Neurosci.*, **13**, 1429-1440.

APRISON, M.H. AND LIPKOWITZ, K.B. (1992). Muscimol and N, N-dimethylmuscimol: from a GABA agonist to a glycine antagonist. *J. Neurosci Res.*, **31**, 166-174.

APRISON, M.H., GALVEZ-RUANO, E., AND LIPKOWITZ, K.B. (1996). Comparison of binding mechanisms at cholinergic, serotonergic, glycinergic, and GABAergic receptors. *J. Neurosci. Res.*, **43**, 127-136.

BOILEAU, A.J. AND CZAJKOWSKI, C. (1999). Identification of transduction elements for benzodiazepine modulation of the GABA$_A$ receptor: three residues are required for allosteric coupling. *J. Neurosci.*, **19**, 10213-10220.

BOILEAU, A.J., EVERS, A.R., DAVIS, A.F. AND CZAJKOWSKI, C. (1999). Mapping the agonist binding site of the GABA$_A$ receptor: evidence for a β-strand. *J. Neurosci.*, **19**, 4847-4854.

BUREAU, M. AND OLSEN, R.W. (1988). γ-Aminobutyric acid/benzodiazepine receptor protein carries binding sites for both ligands on both two major peptide subunits. *Biochem. Biophys. Res. Comm.*, **153**, 1006-1011.

BURT, D. AND KAMATACHI, G.L. (1991). $GABA_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.*, **5**, 2916-2923.

CASALOTTI, S.O., STEPHENSON, F.A. AND BARNARD, E.A. (1986). Separate subunits for agonist and benzodiazepine binding in the γ-aminobutyric $acid_A$ receptor oligomers. *J. Biol. Chem.*, **261**, 15013-15016.

CHANG, Y. AND WEISS, D.S. (1999). Channel opening locks agonist onto the $GABA_C$ receptor. *Nature Neurosci.*, **2**, 219-225.

CLEMENTS, J.D. (1996). Transmitter timecourse in the synaptic cleft: its role in central synaptic function. *Trends Neurosci.*, **19**, 163-170.

COCKROFT, V.B., OSGUTHORPE, D.J., BARNARD, E.A. AND LUNT, G.G. (1990). Modeling of agonist binding to the ligand-gated ion channel superfamily of receptors. *Proteins: Struc. Func. Genet.*, **8**, 386-397.

COLQUHOUN, D. (1998). Binding, gating, affinity, and efficacy: the interpretation of structure-activity relationships for agonists and the effects of mutating receptors. *Br. J. Pharmacol.*, **125**, 924-947.

CUNNINGHAM, B.C. AND WELLS, J.A. (1989). High-resolution epitope mapping of high-receptor interactions by alanine-scanning mutagenesis. *Science*, **244**, 1081-1085.

DAVIES, M. AND DUNN, S.M.J. (1998). Identification of a unique domain in bovine brain $GABA_A$ receptor that is photoaffinity labelled by $[^3H]$Ro15-4513. *Biochem. Biophys. Res. Comm.*, **246**, 650-653.

DAVIES, M., BATESON, A.N. AND DUNN, S.M.J. (1998). Structural requirements for ligand interactions at the benzodiazepine recognition site of the $GABA_A$ receptor. *J. Neurochem.*, **70**, 2188-2194.

DENG, L., RANSOM, R.W. AND OLSEN, R.W. (1986). [^{3}H]Muscimol photolabels the γ-aminobutyric acid receptor binding site on a peptide subunit distinct from that labeled with benzodiazepines. *Biochem. Biophys. Res. Comm.*, **138**, 1308-1314.

DUNN, S.M.J., DAVIES, M., MUNTONI, A.L. AND LAMBERT, J.J. (1999). Mutagenesis of the rat α1 subunit of the γ-aminobutyric $acid_A$ receptor reveals the importance of residue 101 in determining allosteric effects of benzodiazepine site ligands. *Mol. Pharmacol.*, **56**, 768-774.

EBERT, V., SCHOLZE, P. AND SIEGHART, W. (1996). Extensive heterogeneity of recombinant γ-aminobutyric $acid_A$ receptors expressed in $\alpha_4\beta_3\gamma_2$-transfected human embryonic kidney 293 cells. *Neuropharmacology*, **35**, 1323-1330.

FISHER, J.L. AND MACDONALD, R.L. (1998). The role of an α subtype M2-M3 his in regulating inhibition of $GABA_A$ receptor current by zinc and other divalent cations. *J. Neurosci.*, **18**, 2944-2953.

GALLIVAN, J.D. AND DOUGHERTY, D.A. (1999). Cation-π interactions in structural biology. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 9459-9464.

GALVEZ-RUANO, E., APRISON, M.H., ROBERTSON, D.H. AND LIPKOWITZ, K.B. (1995). Identifying agonistic and antagonistic mechanisms operative at the $GABA_A$ receptor. *J. Neurosci. Res.*, **42**, 666-673.

GALZI, J-L., EDELSTEIN, S.J. AND CHANGEUX, J.-P. (1996). The multiple phenotypes of allosteric receptors mutants. *Proc. Natl. Acad. Sci. U.S.A.*, **93**, 1853-1858.

HORNSTEIN, J. AND AKABAS, M.H. (1998). Location of a high affinity Zn^{2+} binding site in the channel of α1β1 γ-aminobutyric acid$_A$ receptors. *Mol. Pharmacol.*, **53**, 870-877.

IM, H.K., IM, W.B., HAMILTON, B.J., CARTER, D.B. AND VONVOIGTLANDER, P.F. (1993). Potentiation of γ-aminobutyric acid-induced chloride currents by various benzodiazepine site agonists with the α1γ2, β2γ2, and α1β2γ2 subtypes of cloned γ-aminobutyric acid type A receptors. *Mol. Pharmacol.*, **44**, 866-870.

IM, W.B., PREGENZER, J.P., BINDER, J.A. ALBERTS, G.L. AND IM, H.K. (1997). Alterations of the benzodiazepine site of rat α6β2γ2-GABA$_A$ receptor by replacement of several divergent amino-terminal regions with the α1 counterparts. *Br. J. Pharmacol.*, **120**, 559-564.

JACKSON, M.B. (1989). Perfection of a synaptic receptor: kinetics and energetics of the acetylcholine receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2199-2203.

MAKSAY, G. and TICKU, M.K. (1984). Characterization of γ-aminobutyric acid-benzodiazepine receptor complexes by protection against inactivation by group-specific reagents. *J. Neurochem.*, **42**, 1715-1727.

MCKERNAN, R.M., FARRAR, S., COLLINS, I., EMMS, F., ASUNI, A. QUIRK, K. AND BROUGHTON, H. (1998). Photoaffinity labeling of the benzodiazepine binding site of α1β3γ2 γ-aminobutyric acid$_A$ receptors with flunitrazepam identifies a subset of ligands that interact directly with his102 of the α subunit and predicts orientation of these within the benzodiazepine pharmacophore. *Mol. Pharmacol.*, **54**, 33-43.

MIHIC, S.J, , YE, Q., WICK, M.J., KOLTCHINE, V.V., KRASOWSKI, M.D., FINN, S.E., MASCIA, M.P., VALENZUELA, C.F., HANSON, K.K., GREENBLATT, E.P., HARRIS, R.A. AND HARRISON, N.L (1997). Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. *Nature*, **389**, 385-389.

NEWELL, J.G., DAVIES, M. AND BATESON, A.N. (2000a). The use of site-directed mutagenesis, transient transfection and radioligand binding: a method for the characterization of receptor-ligand interactions. *Mol. Biotech.*, **14**, 25-45.

NEWELL, J.G., DAVIES, M., BATESON, A.N. AND DUNN, S.M.J. (2000b). Tyrosine 62 of the γ-aminobutyric acid type A receptor β2 subunit is an important determinant of high affinity agonist binding. *J. Biol. Chem.*, **275**, 14198-14204.

NIELSEN, M., WITT, M.R., EBERT, B.AND KROGSGAARD-LARSEN, P. (1995). Thiomuscimol, a new photoaffinity label for the $GABA_A$ receptor. *Eur. J. Pharmacol.*, **289**, 109-112.

OLSEN, R.W., BERGMAN, M.O., VAN NESS, P.C., LUMMIS, S.C., WATKINS, A.E., NAPIAS, C. AND GREENLEE, D.V. (1981). γ-Aminobutyric acid receptor binding in mammalian brain: heterogeneity of binding sites. *Mol. Pharmacol.*, **19**, 217-227.

O'SHEA, S. AND HARRISON, N.L. (2000). Arg-274 and Leu-277 of the γ-aminobutyric acid type A receptor α_2 subunit define agonist efficacy and potency. *J. Biol. Chem.*, **275**, 22764-22768.

PREGENZER, J.F., IM, W.B., CARTER, D.B. AND THOMSEN, D.R. (1993). Comparison of interactions of [^{3}H]muscimol, *t*-butylbicyclophosphoro[^{35}S]thionate and [^{3}H]flunitrazepam with cloned γ-aminobutyric $acid_A$ receptors and of the $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ subtypes, *Mol. Pharmacol.*, **43**, 801-806.

RAFTERY, M.A., DUNN, S.M.J., CONTI-TRONCONI, B.M., MIDDLEMAS, D.S. AND CRAWFORD, R.D. (1983). The nicotinic acetylcholine receptor: subunit structure, functional binding sites, and ion transport properties. *Cold Spring Harb. Symp. Quant. Biol.*, **48**, 21-33.

SIGEL, E., BAUR, R., KELLENBERGER, S. AND MALHERBE, P. (1992). Point mutations affecting antagonist and agonist dependent gating of GABA$_A$ receptor channels. *EMBO J.*, **11**, 2017-2023.

SIGEL, E. AND BUHR, A. (1997). The benzodiazepine binding site of GABA$_A$ receptors. *Trends Pharmacol. Sci.*, **18**, 425-429.

SLANY, A., ZEZULA, J., FUCHS, K. AND SIEGHART, W. (1995). Allosteric modulation of [^{3}H]flunitrazepam binding to recombinant GABA$_A$ receptors. *Eur. J. Pharmacol. (Mol. Pharmacol. Sect).*, **291**, 99-105.

SMITH, G.B. AND OLSEN, R.W. (1994). Identification of [^{3}H]muscimol photoaffinity substrate in the bovine γ-aminobutyric acid$_A$ receptor α subunit. *J. Biol. Chem.*, **269**, 20380-20387.

UNWIN, N. (1995). Acetylcholinie receptor channel imaged in the open state. *Nature*, **373**, 37-43.

WARD, W.H.J., TIMMS, D. AND FERSCHT, A.R. (1990). Protein engineering and the study of structure-function relationships in receptors. *Trends Neurosci.*, **11**, 280-288.

WESTH-HANSEN, S.E., RASMUSSEN, P.B., HASTRUP, S., NABEJURA, J., NOGUCHI, K., AKAIKE, N., WITT, M.-R. AND NIELSEN, M. (1997). Decreased agonist sensitivity of human GABA$_A$ receptors by an amino acid variant, isoleucine to valine, in the α1 subunit. *Eur. J. Pharmacol.*, **329**, 253-257.

WONG, G., SEI, Y. AND SKOLNICK, P. (1992). Stable expression of type I γ-aminobutyric acidA/benzodiazepine receptors in a transfected cell line. *Mol. Pharmacol.*, **42**, 996-1003.

YAN, D., SCHULTE, M.K., BLOOM, K.E. AND WHITE, M.W. (1999). Structural features of the ligand-binding domain of the serotonin $5HT_3$ receptor. *J. Biol. Chem.*, **274**, 5537-5541.

YANG, J.S.-J. AND OLSEN, R.W. (1987). γ-Aminobutyric acid receptor binding in fresh mouse brain membranes at 22°C: ligand-induced changes in affinity. *Mol. Pharmacol.*, **32**, 266-277.